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# Optical biosensor analysis of the heat denaturation of bovine lactoferrin

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**Abstract**

There is growing interest in bovine lactoferrin as a nutritional ingredient, and since thermal exposure may compromise protein functionality, the heat-induced denaturation of native bovine lactoferrin was monitored by a recently developed optical biosensor-based immunoassay. Kinetic parameters were determined between 70 and 90 °C, which confirmed that thermal exposures typical of pasteurisation were relatively benign with respect to retained conformational integrity, while higher temperature–time combinations resulted in significant loss of conformationally intact lactoferrin. The influence of solution pH was also investigated and the biosensor technique compared with an HPLC procedure. The described biosensor immunoassay provides a useful complement to current analytical methods for investigation of heat-induced protein denaturation.


*Keywords:* Lactoferrin; Denaturation; Biosensor

## 1. Introduction

The expression of biological activity for a protein is critically dependent on its three-dimensional folded structure, and the native state is, therefore, considered to correspond to the most thermodynamically stable conformation accessible under those physiological conditions where the protein displays biological function (Price, 2000). Studies of protein stability have generally been based on chemical or thermal denaturation data experimentally obtained by monitoring protein unfolding and potential aggregation through UV circular dichroism, tryptophan-specific fluorescence spectroscopy, gel electrophoresis and related Western blot techniques. The conformational integrity of a biologically active protein and its resistance to denaturation is of increasing significance with the trend towards utilising individual protein isolates therapeutically in foods.

Lactoferrin (Lf) is a ca. 80 kDa basic (pI: ca. 9.0) iron-binding, bi-lobal secretory transport glycoprotein of known amino acid sequence, and is a member of the transferrin family characterised by a carbonate-dependent, high affinity ($K_D \sim 10^{-20}$ M) and reversible binding of two $Fe^{3+}$ per molecule yielding a pink complex (Anderson, Baker, Norris, Rumball, & Baker, 1990; Baker & Baker, 2004; Lonnerdal & Atkinson, 1995, chapter 5; Steijns & van Hooijdonk, 2000; Wong, Camirand, & Pavlath, 1996). Although present in milk as a result of in situ synthesis within the mammary gland, it is also present in several other exocrine fluids. In addition to its antimicrobial activity, Lf may function in intestinal iron uptake and regulation, immune response, growth factor activity, bone growth and antioxidant activity (Cornish et al., 2004; Fox & Kelly, 2003; Pakkanen & Aalto, 1997; Steijns & van Hooijdonk, 2000; Stevenson & Knowles, 2003; Wong et al., 1996). Lf content is species-dependent, with significantly higher levels in human milk and colostrum compared to the bovine equivalent, which has stimulated an increasing trend of supplementation of bovine milk-based infant formulas with fractionated bovine Lf, despite a

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69% primary sequence homology with human Lf (Baker & Baker, 2004; Pakkanen & Aalto, 1997; Steijns & van Hooijdonk, 2000; Stevenson & Knowles, 2003; Wong et al., 1996).

The heat stability, and hence conformational integrity of bovine Lf, is clearly an important factor in determining the efficacy of humanised infant formulas and other milk products supplemented with this protein. Previous studies of the denaturation of Lf have variously targeted native, apo- and holo-forms in either aqueous, buffered or milk systems utilising high performance liquid chromatography, radial immunodiffusion, electrophoresis, antibacterial activity, differential scanning calorimetry and iron-binding capacity techniques (Abe et al., 1991; Kulmyrzaev, Levieux, & Dufour, 2005; Kussendrager, 1994; Luf & Rosner, 1997; Oria, Ismail, Sanchez, Calvo, & Brock, 1993; Paulsson, Svensson, Kishore, & Naidu, 1993; Sanchez et al., 1992). Comparable techniques have also been utilised for both native and recombinant human Lf under consideration for infant formula supplementation (Suzuki et al., 2003).

Immunoassay-based techniques utilising antibodies specific for native, intact and biologically active Lf represent an alternative means to monitor the structural sensitivity of Lf to heat stress, with ELISA techniques described (Suzuki et al., 2003). A relatively recent technology that facilitates the direct, real-time non-labelled detection of proteins is based on the optical phenomenon of surface plasmon resonance (SPR). This biosensor-based technique has been most commonly utilised to study the mechanisms of protein interactions, and kinetic parameters for the interactions of Lf with oocyte receptor, lipopolysaccharide receptor CD14, salivary agglutinin and nucleolin receptor have been reported (Baveye et al., 2000; Hiesberger et al., 1995; Legrand et al., 2004; Mitoma, Oho, Shimzaki, & Koga, 2001). However, SPR-based biosensor immunoassay has also been increasingly utilised for concentration analysis, and the quantitation of bovine Lf content in milk recently reported (Indyk & Filonzi, 2005). This technique has, therefore, been employed to monitor the effect of heat treatment on the conformational stability of bovine Lf in comparison with an independent HPLC procedure.

## 2. Materials and methods

### 2.1. Materials

Bovine lactoferrin was commercially isolated in its native form from pasteurised skim milk by SP Sepharose™ cation exchange liquid chromatography and step elution with salt buffers, and recovered Lf further purified by ultrafiltration for use as a primary calibrant, with identity and purity evaluations as described previously (Cornish et al., 2004; Indyk & Filonzi, 2005) and iron-saturation status typically confirmed as ca. 15%. A stock Lf solution (10 mg/mL) was prepared in water and stored at 4 °C.

Water used was >18 MΩ resistivity. All reagents used for biosensor assay are as described previously (Indyk & Filonzi, 2005), with analysis on a Biacore® Q optical biosensor instrument (Biacore AB, Uppsala, Sweden). These included 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide–HCl (EDC, 0.4 M), *N*-hydroxysuccinimide (NHS, 0.1 M), ethanolamine–HCl (1 M, pH 8.5), sodium acetate buffer (10 mM, pH 5), Sensor Chip CM5 and HBS running buffer (10 mM Hepes, 150 mM NaCl, 3.4 mM EDTA, 0.005% surfactant P20, pH 7.4) obtained from Biacore AB. Affinity purified goat polyclonal anti-bovine Lf (1 mg/mL) was obtained from Bethyl Laboratories (A10-126A) and glycine (AR grade) was obtained from BDH (Poole, UK).

The sensor surface was prepared by immobilising anti-bovine Lf via amine coupling. Briefly, the designated flow cell was activated with a mixture of EDC and NHS (1:1 v/v) (10 μL/min, 7 min) followed by antibody (50 μg/mL in 10 mM sodium acetate, pH 5.0) (10 μL/min, 7 min). Finally, unreacted ester functionalities were deactivated with ethanolamine (1 M, pH 8.5, 10 μL/min, 7 min).

Analysis by reversed-phase HPLC involved the use of an LC10 low-pressure gradient system (Shimadzu, Tokyo, Japan), a C4 column (Asahipak C4P-50 4D) and UV detection at 280 nm as described previously (Abe et al., 1991). Mobile phase A comprised aqueous NaCl (2.7%):acetonitrile (10%):TFA (0.0275%) and mobile phase B comprised aqueous NaCl (1.5%):acetonitrile (50%):TFA (0.015%).

### 2.2. Methods

#### 2.2.1. Heat treatment

In order to investigate the influence of solution pH, Lf (100 μg/mL) was prepared in unbuffered aqueous solution and in water adjusted to pH 4.3 and 9.4, and aliquots (5 mL) placed in a temperature-controlled water-bath at 70 °C. At time intervals, aliquots were diluted 1:100 v/v in HBS buffer containing 0.5 M sodium chloride ready for biosensor immunoassay. Filtration was unnecessary prior to injection even at alkaline pH, where a slight turbidity was observed.

To evaluate the influence of temperature, aliquots (5 mL) of Lf (100 μg/mL) in unbuffered aqueous solution were placed in a temperature-controlled water-bath at 70, 80 and 90 °C and analysed for intact Lf as described above.

A raw skim milk containing high levels of endogenous Lf (~500 μg/mL) was subjected to heat treatment at 70 °C and at the same time intervals was diluted 1:2000 v/v in HBS buffer containing 0.5 M sodium chloride ready for biosensor immunoassay.

For comparative HPLC analysis, a significantly higher concentration of Lf was required due to the inherently low sensitivity of UV detection. Aqueous unbuffered Lf (10 mg/mL) aliquots were subjected to heat treatment at 80 °C. At time intervals, aliquots were withdrawn and diluted 1:5 v/v in 3% aqueous sodium chloride ready for HPLC analysis. These extracts were further diluted 1:2000 v/v for parallel biosensor analysis.

*2.2.2. Biosensor analysis*

Analysis of Lf utilised a goat anti-bovine Lf immobilised sensor surface and validated run conditions as previously described (Indyk & Filonzi, 2005). Briefly, calibration was established by serial dilution of authentic 1000 ng/mL bovine Lf in HBS (0.5 M NaCl). Duplicate calibrants and sample extracts in HBS (0.5 M NaCl) were dispensed into 96-well microtitre plates and 50 μL injected at 10 μL/min with HBS running buffer. Relative binding responses were used both for generation of a 4-parameter fitted calibration curve and interpolation of unknown samples. The surface was regenerated with 35 μL of 10 mM glycine–HCl, pH 1.75 at 50 μL/min.

*2.2.3. HPLC analysis*

Analysis of Lf by HPLC was essentially identical to that previously described (Abe et al., 1991). Briefly, 25 μL was injected under gradient conditions at 0.65 mL/min, with Lf detected at 280 nm.

## 3. Results

Fig. 1 illustrates a typical biosensor analysis obtained for both isolated Lf in aqueous non-buffered solution, and endogenous native Lf in intact skim milk at 70 °C.

Raw data are presented in the form of superimposed sensorgrams of treated Lf at each time interval. Each individual sensorgram monitors the change of SPR signal during contact of Lf over the anti-Lf immobilised surface. The binding response acquired relative to initial baseline is a measure of intact, undenatured Lf remaining in solution.

In order to evaluate the influence of solution pH, native Lf was subjected to heat treatment at 70 °C under neutral, acidic and alkaline pH conditions (a slightly visible turbidity was noted at high pH at $t_0$) and the comparison of residual immunologically intact Lf shown in Fig. 2.

While Lf concentration was unaffected by solution pH at $t_0$, there was a significant effect of pH on the heat stability of Lf, with a dramatic reduction in stability to denaturation under alkaline conditions. Also illustrated is the denaturation of endogenous Lf in intact milk at physiological pH. Both in neutral aqueous solution and in milk at pH 6.8, more than 85% of antigenically intact Lf remained after 2 min at 70 °C.

Fig. 3 illustrates the chromatography obtained for native Lf at 80 °C in water. Significantly higher initial concentrations of Lf were necessary as a consequence of the much lower sensitivity of UV detection compared to SPR.

Prolonged exposure to thermal stress clearly results in a progressive broadening of the Lf peak elution profile to include closely co-eluting denatured forms of the protein. Residual Lf, as estimated by both HPLC peak area and height, was compared against SPR measurement from the same heat treated sample and the data shown in Fig. 4.

It is apparent that there is close equivalence in the estimation of undenatured Lf between chromatographic peak apex response at 280 nm and SPR binding signal.

The denaturation of isolated Lf at neutral pH in water, as measured by SPR-biosensor assay, was predictably dependent on increasing temperature as illustrated graphically in Fig. 5, based on a first-order kinetic model of residual intact Lf with time.

The values for the rate constants $k$ derived at the three temperatures calculated from the least squares linear regression slopes are summarised in Table 1, along with the rate constant estimated for endogenous Lf in milk at 70 °C.

The temperature dependence of the rate constant may be described by the Arrhenius equation ($k = Ae^{-E_a/RT}$), whereby the slope of the linear regression of the natural logarithm of $k$ as a function of reciprocal absolute temperature allows an estimate of the activational energy ($E_a$). This procedure yielded an $E_a$ of 73.8 kJ/mol for the heat denaturation of native Lf isolate in water between 70 and 90 °C.

## 4. Discussion

A protein may exist as a set of related conformations whose interconversion involves the making and breaking of the weak non-covalent interactions that stabilise the folded structure, with the native state considered as a time-average of closely related and thermodynamically most stable conformations (Price, 2000). Thermal denaturation initiates the potentially reversible and first-order unfolding of a protein, and may occur through a mechanism involving partially unfolded ''molten-globule'' intermediate states that can result irreversibly in higher-order aggregation and precipitation (Chen et al., 2005; Kussendrager, 1994; Lonnerdal & Lien, 2003).

Unlike caseins, which are essentially devoid of higher-order structure, whey proteins are relatively heat labile at temperatures above 60 °C. In order to maintain conformational integrity, functional properties and biological activity, it is clearly important to minimise exposure of milk proteins to heat during conventional dairy processing, since whey protein denaturation is largely determined by temperature and holding time. There have, therefore, been many studies related to evaluating the specific heat stability of either isolated bovine milk proteins, most commonly β-lactoglobulin and α-lactalbumin, or intact milk under both extreme thermal conditions as well as those typical of dairy processing (eg Chen et al., 2005; Considine, Patel, Singh, & Creamer, 2005; Kulmyrzaev et al., 2005).

The thermal stability of bovine Lf has generally been studied in model buffered aqueous solutions or when added to milk. The described SPR analysis of residual antigenic determinants over immobilised anti-bovine Lf has shown that native Lf is significantly more resistant to thermal denaturation at pH ca. 4 relative to higher pH, concurring with a previous study of apo-Lf using immunoelectrophoretic and HPLC techniques (Abe et al., 1991), but in contrast to a study utilising differential scanning calorimetry, where native Lf was reportedly more thermostable at basic

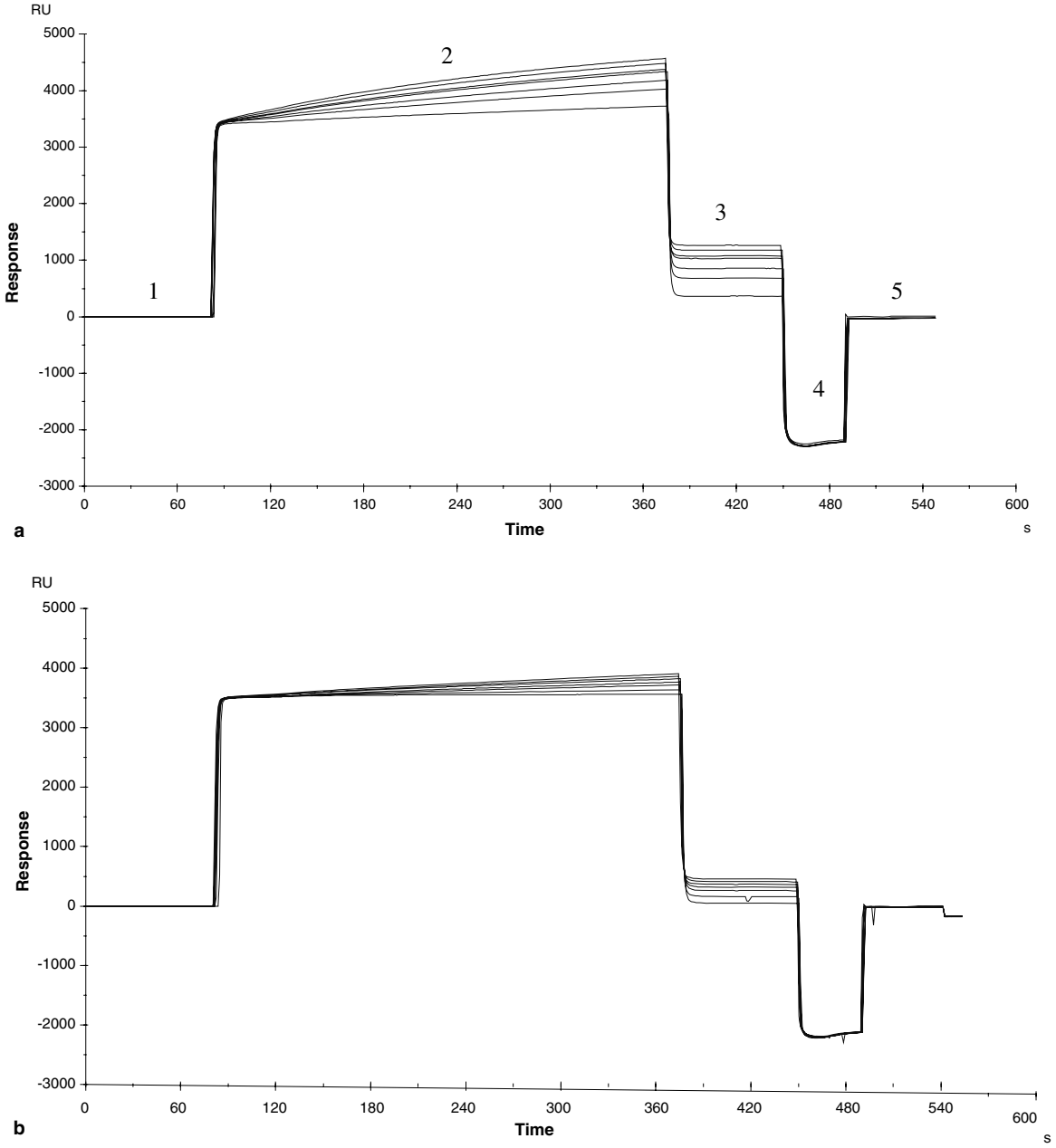


Fig. 1. Overlaid sensorgrams illustrating the binding of residual intact bovine Lf, after heating at 70 °C (0, 2, 5, 10, 30, 60, 160 min), to immobilised goat anti-bovine Lf. 50 μL injected at 10 μL/min. Captions indicate (1) baseline, (2) association (3) relative binding level, (4) regeneration and (5) baseline. (a) Lf isolate and (b) endogenous milk Lf.

pH (Paulsson et al., 1993). The correlation between denaturation kinetics as followed by SPR and HPLC in the present study suggests that the conformational Lf population eluting at the peak apex volume is antigenically intact, while the significant chromatographic peak broadening observed with time plausibly represents conformationally compromised states of Lf with respect to epitope integrity.

The kinetic parameters determined for the heat-induced denaturation of native bovine Lf estimated in water at neutral pH by the SPR immunoassay technique have confirmed that protein unfolding is a first-order process, in accordance with previous studies (Kussendrager, 1994; Sanchez et al., 1992). Measured rate constants, $k$, over the temperature range 70–90 °C are in general agreement with a previous report based on radial immunodiffusion of holo- and apo-Lf in 0.1 M phosphate at pH 7.4 containing 0.15 M sodium chloride (Sanchez et al., 1992). In contrast to the latter study, SPR analysis revealed similar measured $k$ values for endogenous milk Lf and Lf isolate in a neutral pH aqueous system, a result possibly consistent with native Lf of equivalent partly iron-saturated status in both systems. It should be noted that $D$ (time for 90% denaturation) and $Z$ (temperature change to reduce $D$ by 1 log) values were not estimated in this limited temperature

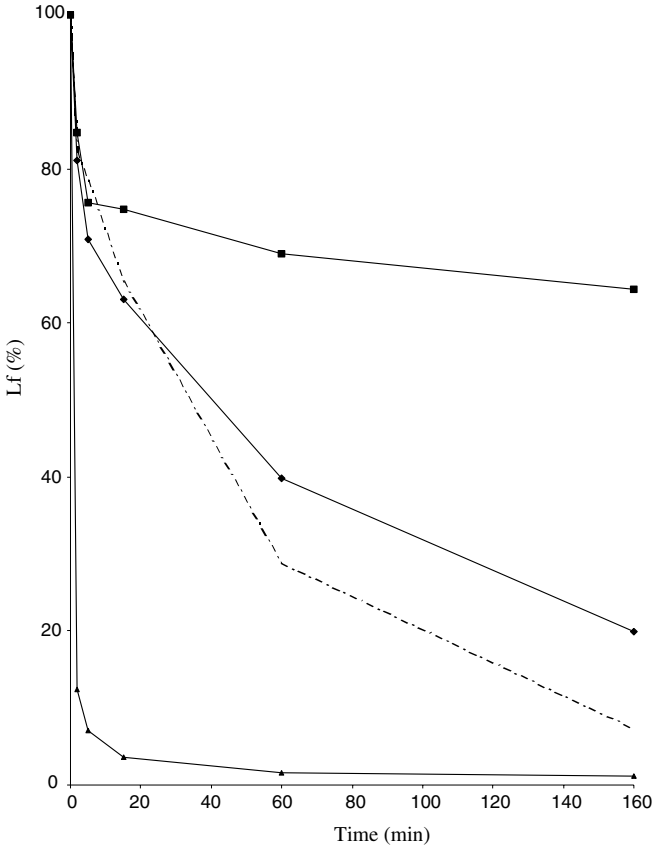


Fig. 2. Residual antigenically active Lf after exposure at 70 °C at pH 7 (◆), 4.3 (■), 9.4 (▲) and in intact milk at pH 6.8 (---). Each data point is the mean of duplicate measurements (RSDr < 2.0%).

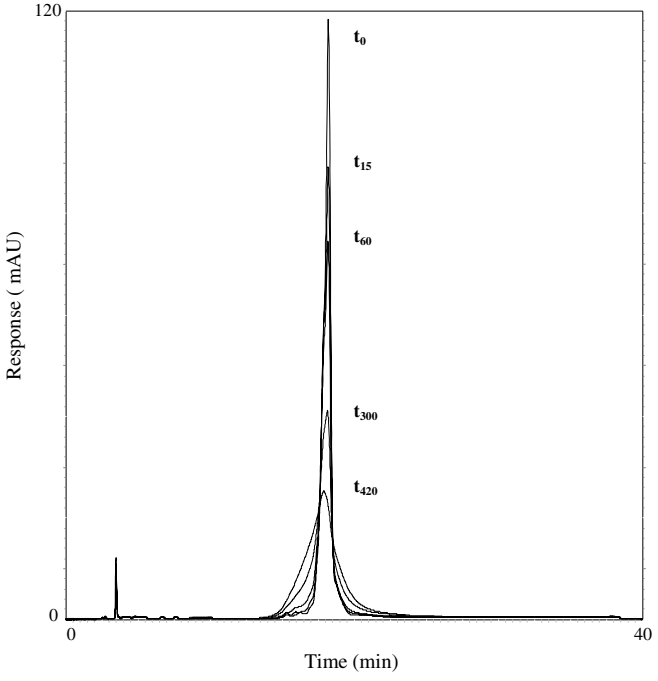


Fig. 3. Overlaid chromatography of Lf heated at 80 °C. Conditions as described in text, detection: 280 nm. Increasing exposure times correspond to progressive peak broadening (*t*: 0, 15, 60, 300, 420 min).

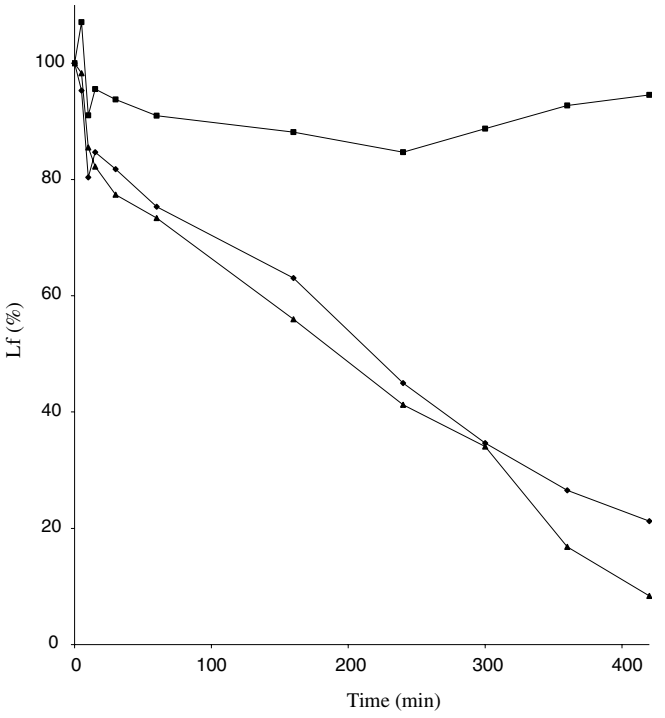


Fig. 4. Residual Lf at neutral pH after heating at 80 °C estimated by HPLC peak area (■), peak height (◆) and SPR (▲).

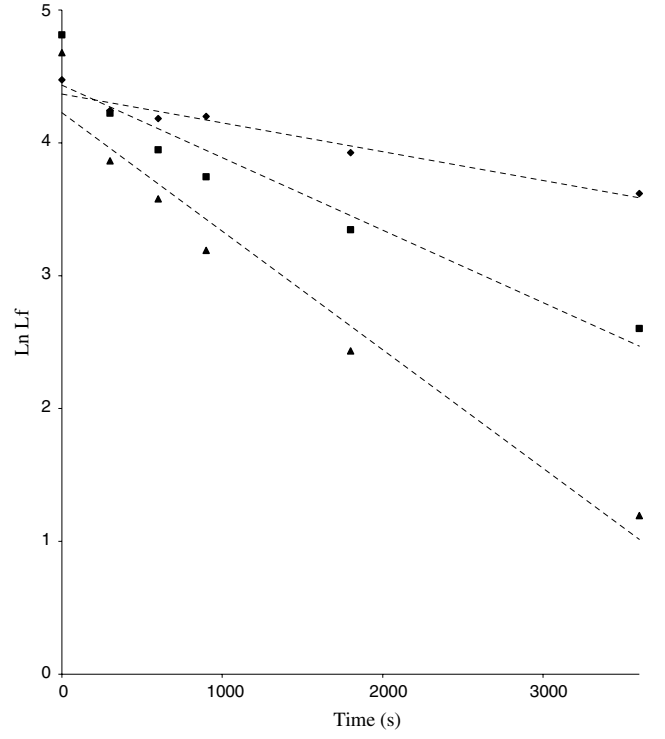


Fig. 5. Denaturation of native Lf in water at neutral pH at 70 °C (◆), 80 °C (■) and 90 °C (▲). Ln Lf is the natural logarithm of residual Lf at each time interval. Each data point is the mean of duplicate measurements (residual Lf RSDr < 3%).

Table 1
Kinetic rate constants ($k \times 10^4\ s^{-1}$) estimated for the denaturation of native Lf

| Temperature (°C) | Lf (isolate) | Lf (milk) |
|---|---|---|
| 70 | 2.5 (0.950)[a] | 3.0 (0.983) |
| 80 | 6.5 (0.917) | nd |
| 90 | 10.3 (0.953) | nd |

nd, not performed.
[a] Correlation coefficient $r^2$ in parentheses.

interval study. A differential scanning calorimetric study indicated somewhat higher $k$ values, although differences in experimental conditions with respect to iron saturation of Lf, concentration, pH, ionic strength and other proteins will undoubtedly influence thermal stability and hence measured kinetic parameters (Kussendrager, 1994). The activation energy of Lf denaturation as determined by the described SPR immunoassay is lower than previously reported values (Sanchez et al., 1992), although consistent with the 50–200 kJ/mol typical of many proteins (Alichanidis, Wrathall, & Andrews, 1986; Price, 2000).

There is evidence that extent of Lf denaturation is strongly influenced by its iron-saturation status. Thus, apo- and holo-Lf are denatured at different rates, reflective of the profound conformational change upon iron-binding, and further, that the heat-induced unfolding of the two lobes of native Lf are independent (Baker & Baker, 2004; Kussendrager, 1994; Paulsson et al., 1993; Sanchez et al., 1992). A recent investigation using RID has shown that endogenous bovine milk whey proteins were increasingly stable to denaturation between 57 and 72 °C in the order alkaline phosphatase, Lf, IgG, BSA, β-Lg, α-La, although no kinetic data were reported (Kulmyrzaev et al., 2005), while a study of human Lf (native and recombinant) has similarly evaluated the influence of heat exposure on biological activity (Suzuki et al., 2003).

The present study focussed on native Lf denaturation, since it is in this form that bovine Lf is subjected to thermal processing during both its commercial isolation and the production of bovine milk products intended for infant nutrition (Luf & Rosner, 1997; Steijns & van Hooijdonk, 2000). The described immunoassay based on SPR detection follows heat-induced protein denaturation via the loss of conformational integrity of the multiple epitope determinants of native Lf recognised by an immobilised polyclonal antibody. Similar SPR-based biosensor immunoassay techniques have also recently been applied to the denaturation of both milk folate-binding protein and α-lactalbumin, in view of their potential utility as indicators of heat treatment (Dupont, Rolet-Repecaud, & Muller-Renaud, 2004; Indyk & Filonzi, 2004; Nygren, Sternesjö, & Björck, 2003).

## 5. Conclusions

In general, protein denaturation may be partial, where conformation may be disturbed yet retain functionality, or extensive, leading ultimately to aggregation, precipitation and loss of function. There is no single method for estimating protein denaturation and rate and extent will be dependent on the method of analysis selected. SPR immunoassay has been demonstrated to be a valuable technique to complement existing methods and has confirmed that, at normal physiological pH, conventional pasteurisation conditions are benign with respect to Lf denaturation, while loss of biological functionality is predictably extensive at higher temperatures and longer exposure times.

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