

Immunoprobes for Thermally-induced Alterations in Whey Protein Structure and their Application to the Analysis of Thermally-treated Milks

LUDMILA KARAMONOVÁ,¹ LADISLAV FUKAL,¹ MILAN KODÍČEK,¹ PAVEL RAUCH,¹ E. N. CLARE MILLS² AND MICHAEL R. A. MORGAN^{2*}

¹Department of Biochemistry and Microbiology, Institute of Chemical Technology, 16628 Prague 6, Czech Republic; ²Institute of Food Research, Norwich Research Park, Colney, Norwich NR4 7UA, UK

(Original manuscript received 20 July 2003; revised manuscript accepted 15 January 2004)

Polyclonal antisera have been raised to the whey proteins α -lactalbumin [α -La] and β -lactoglobulin [β -Lg], variants A and B. These antibody preparations have been used to develop enzyme-linked immunosorbent assays (ELISAs) for each of these proteins, which had limits of detection of 13 ng/ml [α -La], 27 ng/ml [β -Lg, variant A], and 20 ng/ml [β -Lg, variant B]. The α -La ELISA did not show any cross-reaction with β -Lg, and neither of the β -Lg ELISAs showed a cross-reactivity with α -La. However, despite the almost identical sequences of variants A and B of β -Lg, the variant A ELISA had a cross-reactivity of 66% with variant B, whilst the variant B ELISA had a cross-reactivity of more than 200% with variant A. The effect of thermal treatment on the immunoreactivity of purified whey proteins was studied by ELISA and related to changes in secondary and tertiary structure determined using CD and fluorescence spectroscopy. The immunoreactivity of α -La determined by ELISA decreased on heating above 90°C, these changes coinciding with the protein denaturation as indicated by a loss of secondary structure. In contrast, the ELISA immunoreactivity of both β -Lg variants increased after heating, a change that also coincided with changes in β -Lg secondary and tertiary structure as determined by intrinsic fluorescence of the protein. Similar thermally-induced changes in whey protein immunoreactivity were observed following heat-treatment of raw milk, the immunoreactivity of α -La being reduced whilst that of the β -Lg variants increased. When used in combination these ELISAs were able to discriminate between milks which had been pasteurized or subjected to more severe heat-treatments such as sterilization and ultra heat treatments (UHT). These data demonstrate that such immunoassays have the potential to be used as quality control methods for determining the thermal history of milks.

*Current address: Procter Department of Food Science, University of Leeds, Leeds LS2 9JT, UK
Correspondence: Professor Pavel Rauch, Department of Biochemistry and Microbiology, Institute of Chemical Technology, 16628 Prague 6, Czech Republic; E-mail: Pavel.Rauch@vscht.cz

Keywords: β -lactoglobulin, α -lactalbumin, secondary structure, immunoassay, heat-treatment

INTRODUCTION

Heat treatments are widely used by the dairy industry, primarily as a means of preservation. This includes mild treatments, such as pasteurization where milk is heated to 72–84°C/15–20 seconds followed by rapid cooling to 4°C, more severe treatments such as ultrahigh temperatures (UHT) where milk is heated to 130–150°C for very short periods of time (e.g. 4–5 s), or sterilization where milks are heated above 115°C for longer periods of up to 15 min. The timing and intensity of the heat treatments is important to ensure the destruction of potential food pathogens, thus ensuring the microbiological safety of processed milks. However, such thermal processing also has the potential to cause extensive changes to the structure of milk proteins, as it results in varying degrees of unfolding and aggregation (Clark, 1998), together with covalent modifications such as glycation, and lactosylation (Leonil *et al.*, 1997).

The major proteins found in milk are caseins and whey proteins. The caseins are arranged into very large micellar structures in milk, comprising α - and β -caseins held together by the less abundant κ -casein. Whey is comprised primarily of the globular proteins α -lactalbumin (α -La) and β -lactoglobulin (β -Lg), together with minor constituents such as bovine serum albumin and immunoglobulin (Walstra & Jenness, 1984). α -La is a 14,200 dalton calcium binding protein which has a role in regulating lactose synthase (Brew & Grobler, 1992). Its three dimensional structure is primarily α -helical in nature with some 3_{10} helix and β -sheet with the calcium binding site being the most ordered part of the protein structure. At low pH or moderately elevated temperatures α -La exists in a partially- folded, or 'molten globule' state (Baum *et al.*, 1989). β -Lg is an 18,400 dalton retinol binding protein with a β -barrel structure characteristic of the lipocalin superfamily (Brownlow *et al.*, 1997). It is present as a mixture of monomers and dimers at neutral pH, the proportion of monomers increasing on heating to 70°C (Aymard *et al.*, 1996) and it had been suggested that it forms a partially folded intermediate following thermal and alkaline pH induced denaturation (Casal *et al.*, 1988).

On heating milk α -La and β -Lg become associated with the casein micelles, β -Lg initiating this process and becoming non-covalently linked via hydrophobic interactions and intermolecular disulphide bonds to κ -casein (Smits & van Brouwershaven, 1980), variant B reacting much faster than variant A (McKenzie *et al.*, 1971). Such interactions affect milk protein properties, such as solubility, foaming, gelation and emulsification, in a poorly controlled manner. In some instances, the alterations are beneficial, e.g. the rheological properties of fermented milk products such as yoghurts are improved if milks are heated prior to manufacture. As thermal processing has such an impact on the functional properties of milk proteins, there is a need for methodology able to provide information on the thermal history of milks and milk products, for process control purposes and to meet food safety regulations.

The standard method for quality control of pasteurization is monitoring the inactivation of milk enzymes, such as alkaline phosphatase and peroxidase (Monget & Laviolette, 1978), whilst lactulose formation has been used as a marker for more severe treatments such as UHT and sterilised milks (IDF, 1991). However, the activities and levels of such components can vary between different batches of milk, independent of any treatment they might have undergone, making such analysis less reliable as a means of determining the thermal history of a given milk sample. They also give no information on the effect of processing history on the protein quality, which may affect milk protein performance in further processing. An alternative approach is to exploit the sensitivity of antibody-binding reactions to alterations in protein structure and aggregation, to indicate the severity of heat-treatment by measuring the degree of thermally-induced alterations in protein structure. Methods such as enzyme-

linked immunosorbent assay (ELISA) also have great potential as a quality control tool, as such methods are easy to use, rapid and with a high sample throughput. ELISAs sensitive to thermal treatments have been developed for several food proteins, including soya globulins (Huang *et al.*, 1998) and the whey protein, α -La (Jeanson *et al.*, 1999) and β -Lg (Negroni *et al.*, 1998). In the latter studies monoclonal antibodies (Mabs) were raised to native and denatured forms of β -Lg. Whilst an immunoassay suitable for identification of severely heated milk samples, including UHT and sterilized milks was developed for α -La, although the β -Lg immunoassay was primarily developed for detection of that protein in non-bovine milks rather than as a method for determining thermal history.

This report describes the development of polyclonal antisera against the whey proteins, α -La and β -Lg, the binding of which are sensitive to thermally-induced alterations in α -La and β -Lg structure, and the application of these antibody preparations to determining the thermal history of processed milks.

MATERIALS AND METHODS

Chemicals, Instruments, Milk

α -LA, β -Lg variants A and B, gelatin and o-phenyldiamine were purchased from Sigma Chemical Co. (St. Louis, USA). Al-span oil and horseradish peroxidase-labelled anti-rabbit IgG (raised in swine) were from Sevac (Prague, Czech Republic). Type U microtitration plates were from Corning Costar Corporation (Cambridge, USA). Prosep A was from Bioprocessing Ltd. (Durham, UK). Raw milk was obtained from a local dairy, pasteurised, UHT and sterilized milks were obtained from local retail outlets.

Heat-treatment of Whey Proteins

Aliquots of α -LA, and β -Lg variants A and B (0.2 mg/ml) in phosphate buffered saline (PBS: 0.14M NaCl, 0.0015M KH_2PO_4 , 0.008M Na_2HPO_4 , 0.0027M KCl; pH 7.4) were heated either at 30, 40, 50, 60, 70, 80 and 90 °C for 10 min in a water bath or at 100, 110, 120, 130 and 140 °C in an oil bath. Samples were then removed and allowed to cool to ambient temperature.

Antibody

Polyclonal antisera were produced to the whey proteins α -LA, and β -Lg variants A and B in New Zealand White rabbits. Protein (0.250 mg) was dissolved in 0.2 ml of PBS and emulsified in 0.2 ml of Complete Freund's adjuvant (Sigma-USA) and protein administered to each animal subcutaneously in the rear legs. After 21 days a booster injection was given in the same manner, but with 0.250 mg of protein was dissolved in 0.16 ml of PBS to which 0.160 ml of Al-Span Oil I and 0.48 ml of Al-Span-Oil II were added and mixed together. This injection was repeated twice at intervals of 21 days before giving a final injection with 0.5 mg of protein emulsified as described for the booster injections. Seven days after the final booster animals were bled by intra-cardial puncture and the blood was centrifuged $1000 \times g$, 10 min, ambient temperature after allowing to stand for 30 min. ϵ -aminocaproic acid and sodium azide were added to 0.03% (w/v) and 0.1% (w/v) respectively and the sera stored at 5 °C.

The IgG fraction of these sera was isolated by affinity chromatography on protein A (Prosep A), dialysed against 0.1M NH_4HCO_3 and the IgG concentration determined spectrophotometrically at 280 nm using an extinction coefficient of 1.4. Lactose was added at a ratio 1:1 (w:w) as a cryoprotectant and fractions lyophilized.

Immunoassay Procedures

Whey proteins were dissolved in 0.05 M-carbonate-bicarbonate buffer, pH 9.6 to 0.4 $\mu\text{g ml}^{-1}$ (α -LA), 0.08 $\mu\text{g ml}^{-1}$ (β -Lg variant A) or 0.025 $\mu\text{g ml}^{-1}$ (β -Lg variant B) and 0.2 ml/well

added to the inner 60 wells of microtitration plates. After incubating for 16 h at 5°C the wells were washed four times with 0.2 ml/well of 0.1 M-PBS-Tween 20 (PBS containing 0.05% (v/v) Tween 20), allowed to dry in air and stored at 5°C. Proteins were diluted in PBST containing 1% (w/v) gelatin to give concentrations ranging between 0.5 ng-500 µg ml⁻¹ and added (0.05 ml/well) to microtitration plates coated with the appropriate protein, together with 0.05 ml/well of protein-specific IgG diluted in the range 1:1,750–1:14,000 (v:v) in PBST containing 1% (w/v) gelatin. After incubation for 1 h at 37°C plates were washed four times with PBST, and 0.1 ml/well added of horseradish peroxidase-labelled anti-rabbit IgG diluted 1:4,000 (v:v) in PBST containing 1% (w/v) gelatin. Plates were then incubated for 1 h at 37°C, and washed a further four times with PBST. Substrate (4.6 mM-o-phenyldiamine in 0.1 M-citrate-phosphate buffer pH 5.0 containing 0.1% (v/v) of a 30% hydrogen peroxide) was then added to each plate (0.1 ml/well). Following a 15 min incubation at 37°C the enzyme reaction was stopped by adding 0.05 ml/well of 2 M-H₂SO₄. The absorbance of each well at 492 nm was then measured using a microplate reader (Multiscan MCC/340, Labsystems, Helsinki, Finland).

Calibration curves were fitted with a four-parameter sigmoidal curve according to equation (1) below:

$$A = C + \frac{D - C}{1 + \exp(-2 \times (\alpha + \beta \times x))} \quad (1)$$

C = value of the bottom asymptote

D = value of the top asymptote

α = constant characterizing the position of the linear nonasymptote of the curve regarding antigen concentration

β = constant characterizing the slope of the linear nonasymptote of the curve

x = common logarithm of antigen concentration

A = absorbance at 492 nm

The top (C) and bottom asymptote (D) were used for calculation of $A_{50\%}$ as follows: $A_{50\%} = (C + D)/2$. The corresponding $c_{50\%}$ was calculated from the equation (2) as follows:

$$c = 10^{(((\ln((D - A)/(A - C)))/(-2)) - \alpha)/\beta} \quad (2)$$

Cross-reactions were calculated as a percentage using equation (3)

$$CR(\%) = \frac{(c_{50\%})_1}{(c_{50\%})_2} \times 100 \quad (3)$$

where $(c_{50\%})_1$ was the concentration at $A_{50\%}$ of immunizing antigen and $(c_{50\%})_2$ the concentration at $A_{50\%}$ of cross-reacting antigen.

Immunoreactivity was calculated as follows. A calibration curve was prepared on each microplate using immunizing antigen diluted in PBST containing 1% (w/v) gelatine. Each sample was diluted in the same buffer to different extents, to give absorbance values at 492 nm close to the $A_{50\%}$ value of the calibration curve. Equation (2) was then used to calculate the apparent concentration of immunizing antigen in each sample. Immunoreactivity was calculated as a percentage using equation (4):

$$IR(\%) = \frac{c_h}{c_n} \times 100 \quad (4)$$

where c_n was the concentration of antigen in native sample (raw milk) and c_h the concentration of antigen in heat treated sample.

Fluorescence and CD Spectroscopy

Fluorescence spectra were recorded using an Aminco-Bowman spectrofluorimeter (Norwalk, CT, USA). Initially the excitation maximum for each protein was determined at an emission wavelength of 340 nm, and found to be 291 nm for α -lactalbumin and 295 nm for β -Lg variants A and B. These were used as the excitation wavelengths for collection of emission spectra. A scan speed of 240 nm/min, slit width of 2.5:2.5 mm and cell path length of 1 cm were used throughout. Samples were 0.2 mg protein per 1 ml of PBS.

CD spectra were recorded using a Jobin-Yvon Autodichrograph Mark V spectropolarimeter (Jobin-Yvon, Longjumeau, France) and analysed using Dichrosoft version A (Jobin-Yvon, Longjumeau, France). Spectra were collected using a scan speed of 0.5 nm s^{-1} using a 1 s time constant, 0.5 nm step resolution, and a sensitivity of 2 millidegrees; the bandwidth was automatically regulated. A path length of 0.1 cm was used and protein samples were 0.2 mg in 1 ml of PBS.

RESULTS AND DISCUSSION

Thermally-induced Alterations in Whey Protein Immunoreactivity

Polyclonal antisera were raised to the whey proteins α -LA and β -Lg (both variants A and B) and their binding specificity towards other whey proteins determined by inhibition ELISA. Typical calibration curves for sera are shown in Figure 1, the α -LA assay being the most sensitive of the three with a limit of detection of 13 ng ml^{-1} (Figure 1A), whilst the β -Lg variant A ELISA had a detection limit of 27 ng ml^{-1} (Figure 1B). The β -Lg variant B ELISA, was slightly more sensitive than that for variant A, with a detection limit of around 20 ng ml^{-1} (Figure 1C). The specificities of the ELISAs are summarized in Table 1, from which it can be seen that the anti- α -LA antiserum was specific for that protein, with no cross-reactivity towards the other whey proteins β -Lg and BSA. Similarly, the anti- β -Lg antisera raised to variants A and B were both β -Lg specific, with no reaction towards other whey proteins. However, some cross-reactivity was observed between the variants, the anti- β -Lg A antiserum giving a cross-reaction of 20 and 66% with variant B and the anti- β -Lg B antiserum giving a cross-reaction of over 200% with variant A. This level of discrimination was surprising in view of the fact that variants A and B have almost identical amino acid sequences, with only two amino acid substitutions between the variants, Asp⁶⁴ and Val¹¹⁸ of variant A being replaced by Gly⁶⁴ and Ala¹¹⁸ respectively in variant B (Bell *et al.*, 1981). However, in a panel of monoclonal antibodies (Mabs) raised to β -Lg A several bound preferentially to variant B in a pH-dependent manner (Kuzmanoff & Beattie, 1991). The Asp⁶⁴Gly substitution is thought to alter the charge distribution in one of the loops of β -Lg (Qin *et al.*, 1999) and it maybe that this is responsible for the discrimination shown by the antibody preparations described in this report.

Solutions of α -LA and both β -Lg variants were then heated to a range of temperatures between 30° – 140°C for 10 min, allowed to cool to room temperature, and their immunoreactivity with these antisera determined by inhibition ELISA (Figure 2). The immunoreactivity of α -LA was reduced by 48% after heating to 100°C , and then abolished after heating above 120°C (Figure 2A). In contrast the immunoreactivity of both β -Lg variants began to change on heating to 50 – 60°C , showing a marked increase in their immunoreactivity when heated to 70°C (Figure 2B,C). Above this temperature, the variants behaved differently from one another. Thus, the immunoreactivity of variant A increased to around 1370% of that observed for the unheated protein, peaking at a temperature of 90°C , whilst that of variant B increased to 18 times that observed for unheated variant B, the immunoreactivity peaking at 80°C .

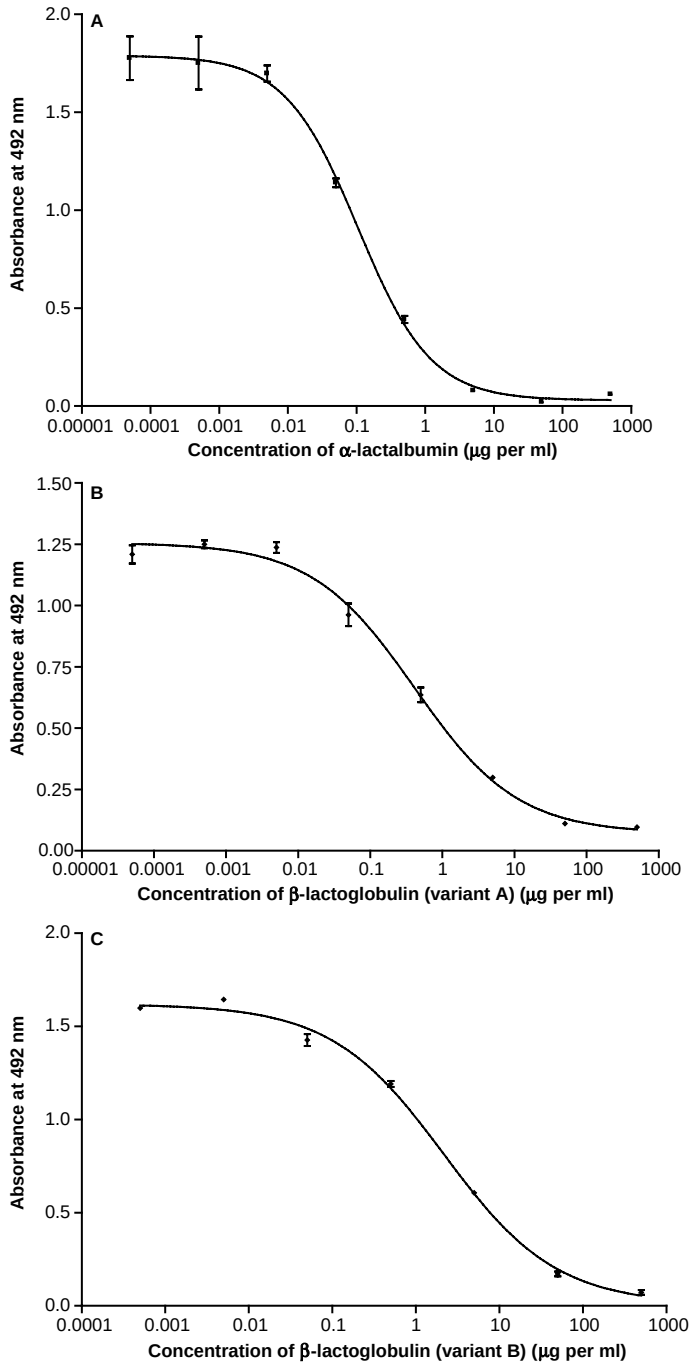


FIG. 1. Inhibition ELISA calibration curves for α -lactalbumin (A), and β -lactoglobulin variants A (B) and B (C).

TABLE 1. Cross-reactivities of polyclonal antisera raised to the whey proteins α -lactalbumin and β -lactoglobulin variants A and B

Antiserum	% Cross-reaction			
	α -Lactalbumin	β -Lactoglobulin variant A	β -Lactoglobulin variant B	Bovine serum albumin
Anti- α -Lactalbumin				
serum 1	100	<0.01	<0.01	<0.01
serum 2	100	<0.01	<0.01	<0.01
Anti- β -Lactoglobulin variant A				
serum 1	<0.01	100	20	<0.01
serum 2	<0.01	100	66	<0.01
Anti- β -Lactoglobulin variant B				
serum 1	<0.1	282	100	<0.1
serum 2	<0.1	245	100	<0.1

Thermally-induced Alterations in Whey Protein Secondary and Tertiary Structure

The relationship between thermally-induced alterations in immunoreactivity and changes in secondary and tertiary structure of whey proteins which were heated and then cooled to room temperature were assessed using intrinsic fluorescence and far-UV CD spectroscopy. Conformational changes, such as those induced by heat-treatments, can affect the emission wavelength ($\lambda_{\max}$) of tryptophan fluorescence and in native β -Lg Trp¹⁹ is thought to be the major fluorophore (Cho *et al.*, 1994; Manderson *et al.*, 1999). The $\lambda_{\max}$ values for both the variants of β -Lg were around 339 nm (Figure 3), a somewhat higher value than those of 332 nm obtained by other workers for the native proteins at pH 7.4 (Manderson *et al.*, 1999). The fluorescence of native α -LA is dominated by that of Trp¹⁰⁴, as the fluorescence of Trp⁶⁰ and Trp¹¹⁸ is largely quenched by the disulfide bonds in their vicinity. These residues have distinct fluorescence maxima of 330, 336 and 339 nm, for Trp¹⁰⁴, Trp⁶⁰ and Trp¹¹⁸ respectively (Chakraborty *et al.*, 2001).

On heating and then cooling the fluorescence maxima ($\lambda_{\max}$) of both whey proteins showed a red shift, moving to longer wavelengths (Figure 3). For α -LA, $\lambda_{\max}$ showed such a shift after heating the protein to a temperature of 80°C, reaching a plateau value of 352.5 nm after heating to 110°C, compared with a $\lambda_{\max}$ of 333 nm for the unheated protein. A similar red shift to a $\lambda_{\max}$ of 351($\pm$ 1 nm) was observed for α -LA which had been unfolded using 8 M-urea (Chakraborty *et al.*, 2001). The $\lambda_{\max}$ value of β -Lg variants A and B also began to increase after heating to 90°C, tailing off at around 110°C to a value of 346 and 347 nm for variant A and B respectively. These results are similar to the values obtained by Manderson *et al.* (1999) for β -Lg heated to 90°C, who also found that variant B was slightly more thermostable than variant A at pH 7.4. There was then a second increase in the $\lambda_{\max}$ values for these proteins after heating to 140°C, to around 348 and 350 nm for β -Lg variants A and B respectively. These values are similar to those obtained by others for the protein in urea (Creamer, 1995), and in thermally-induced aggregates (Carrotta *et al.*, 2001). These data indicate that the Trp residues have become fully exposed to solvent following heating to 140°C.

The effect of heating and then cooling on whey protein secondary structure was also investigated using UV CD spectroscopy (Figure 4). The far UV CD spectrum for α -LA (Figure 4A) is typical of that obtained for an α -helical protein, having a sharp decrease in ellipticity from 200 nm, reaching a negative minimum from 205–210 nm, and then increasing to give a second, less intense, negative minimum from 210–225 nm. Heating α -LA only resulted in spectral changes above 90°C, at which point the negative minimum from 205–210 nm was lost. Continuing the heat treatment to 140°C resulted in a considerable loss of

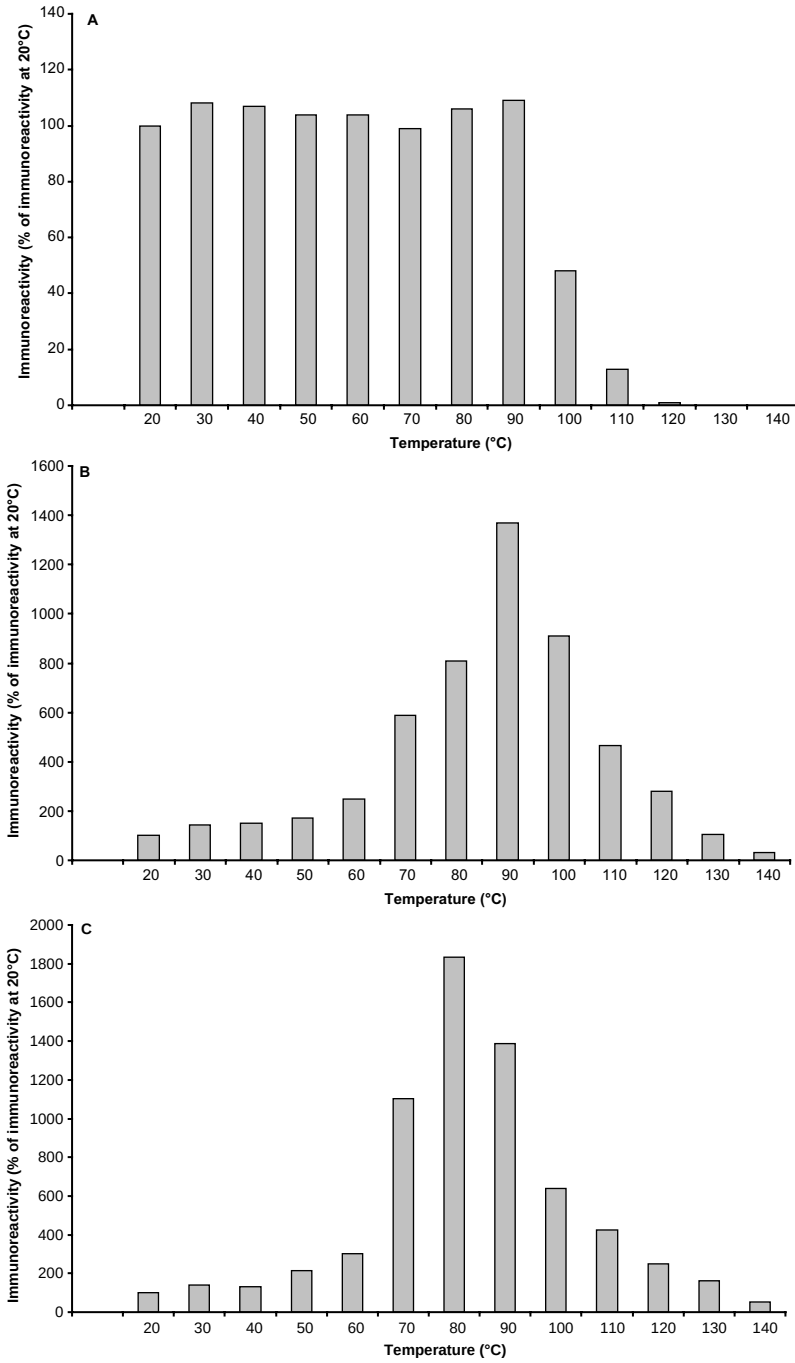


FIG. 2. Effect of heating on the immunoreactivity of α -lactalbumin (A), and β -lactoglobulin variants A (B) and B (C), as determined by inhibition ELISA. Proteins (0.2 mg ml^{-1} in phosphate buffered saline) were heated and then allowed to cool to ambient temperature prior to analysis by the appropriate immunoassay.

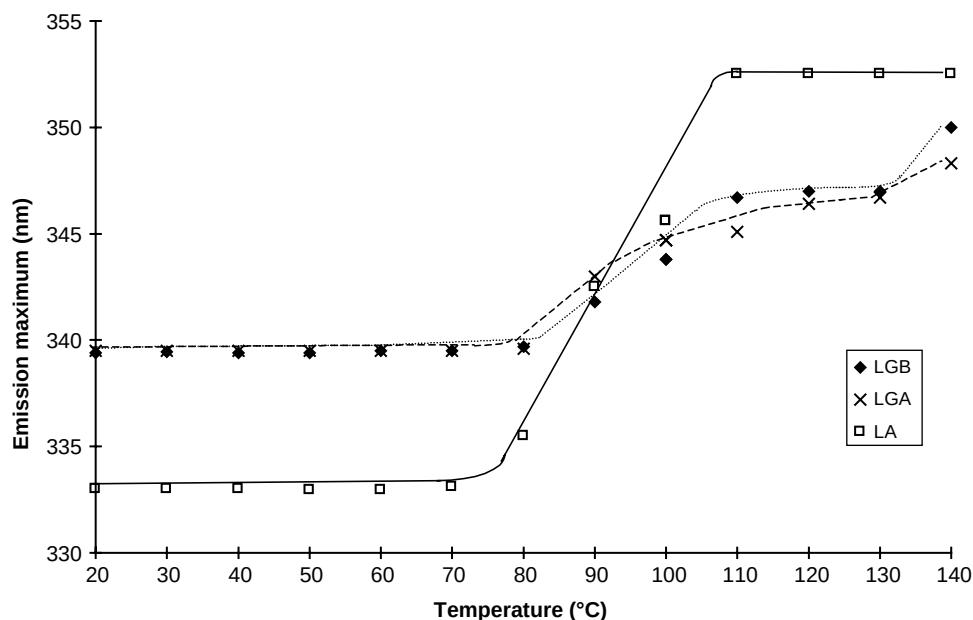


FIG. 3. Effect of thermal-denaturation on the intrinsic fluorescence of α -lactalbumin, and β -lactoglobulin variants A and B. Excitation wavelengths of 291 nm for α -lactalbumin and 295 nm for β -Lg variants A and B were used for collection of emission spectra. Protein solutions were 0.2 mg ml^{-1} in PBS and a scan speed of 240 nm per 1 min, slit width of 2.5:2.5 mm and cell path length of 1 cm were used throughout.

secondary structure, with the loss of the negative minimum between 210–225 nm characteristic of α -helical proteins. After treatment at 110°C the ellipticity also gradually decreased to give a negative minimum at 200–210 nm. This developed into a sharp minimum at 203 nm after treatment at 140°C, which coupled with the increase in ellipticity from 210–250 nm, was indicative of the high content of random coil/ β -structure present in this extensively denatured samples of α -LA.

The far UV CD spectra of β -Lg variants A and B were virtually identical (Figure 4B, C) and characteristic of proteins rich in β -structure. The native protein gave a positive maximum in ellipticity at 200–202 nm, which decreased to give a shallow negative minimum from 208–215 nm, which gradually increase to zero at 250 nm. Heating resulted in no change in the CD spectrum up to 70°C. Above that temperature the positive maximum at 200–202 nm was lost, being replaced by a negative maximum, the minimum becoming more intense and extending from 205–215 nm. The β -Lg spectra changed continually on further heating to 110°C, showing a further shift and intensification of the negative minimum to 200–210 nm, which then gave way to a gradual increase in ellipticity above 210 nm. Whilst there was little further change in the β -Lg spectra following heating to 130°C, although treatment at 140°C resulted in a steeper increase in ellipticity from 210–225 nm.

Immunochemical Analysis of Thermally-treated Milks

The whey protein ELISAs for α -LA and β -Lg were then used to assess the thermally-induced alterations in whey proteins in milk samples. In raw milk the levels of α -LA and β -Lg were found to be around $1\text{--}2 \text{ mg ml}^{-1}$ and compare with the levels of around 1 mg ml^{-1} for α -LA, $2\text{--}3 \text{ mg ml}^{-1}$ for β -Lg commonly found in milk (Walstra & Jennes, 1984). Such a level of agreement regarding the levels of whey proteins in milk might be anticipated as the

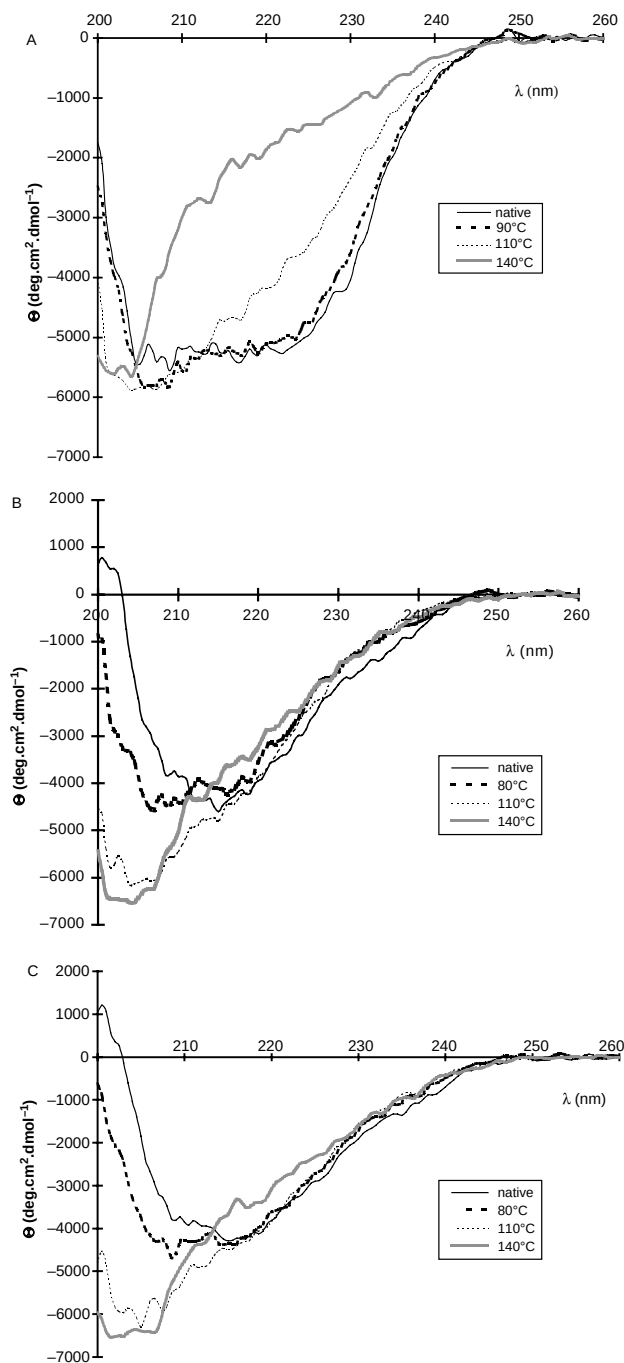


FIG. 4. Far-UV CD spectra of thermally-treated of α -lactalbumin (A), and β -lactoglobulin variants A (B) and B (C). Spectra were collected using a scan speed of 0.5 nm s^{-1} using a 1 s time constant, 0.5 nm step resolution, and a sensitivity of 2 millidegrees; the bandwidth was automatically regulated. A path length of 0.1 cm was used and protein samples were 0.2 mg ml^{-1} in PBS.

polyclonal antisera used in this study were raised to native proteins. These data were taken to represent a baseline for changes in apparent levels of whey proteins in milks following heat-treatment determined by ELISA. The relative changes in antibody binding have been termed "immunoreactivity" and calculated using a value obtained for unheated protein or milk samples as 100%. Thus, the immunoreactivity of a given whey protein in heated milk samples will be determined by both the amount of the individual protein, and the way in which the antibody preparation recognizes the thermally-denatured and aggregated protein.

A set of model heat treatments were employed which simulated conditions used in dairy industry, using purified whey proteins, α -LA and β -Lg variant B, diluted in PBS pH 7.4 (Table 2). Whilst the model pasteurization heat-treatments had only a small effect on α -LA, the more severe UHT treatment reduced its immunoreactivity to around 20% of the native protein, whilst sterilisation almost completely abolished it (Figure 5A). For β -Lg all the heat treatments increased immunoreactivity, with pasteurisation at 85°C for 15 s increasing it by 9-fold, whilst UHT treatment increased it around 8-fold. Even the most severe treatment, sterilization, resulted in a doubling of β -Lg immunoreactivity (Figure 5B).

Based on these results, the ELISAs for α -LA and the β -Lg variant B were chosen to assess their ability to discriminate between various commercially heat-treated milks (Table 3). Following pasteurization the levels of immunoreactive α -LA in milk were almost identical with those found in raw milk, whilst the levels of β -Lg immunoreactivity increased by around 14-fold. In the more severely processed UHT milk α -LA immunoreactivity was reduced to about 18% of that found in raw milk, in contrast to the β -Lg, the reactivity of which declined somewhat compared with the pasteurized milk, but was still whilst more than five times the immunoreactivity of the raw milk. The most severely heat-treated sample was the sterilized milk, in which the α -LA immunoreactivity dropped to only 1% of the raw milk. The immunoreactivity of the milk β -Lg was reduced still further by this treatment, but was still around 140% of that found in raw milk.

GENERAL DISCUSSION

It has been estimated that the majority of epitopes recognized by anti-protein antibodies are conformational (van Regenmortel, 1992), and it is for this reason that antibody-binding reactions may be perturbed by changes in the three-dimensional structure of a protein such as those that accompany thermal denaturation. Thus immunoassays offer a unique means of determining the thermal history of a sample, which is an important aspect of quality control of thermal processes used in the preservation of foods. In an effort to demonstrate the applicability of such methods to determining the thermal history of milk samples, polyclonal antibody preparations were developed to native whey proteins from cow's milk. As might be anticipated the anti- α -LA antiserum preferentially recognized the native, unheated protein, and on heating above 90°C for 10 min the immunoreactivity decrease coincided with the alterations in secondary structure indicative of the α -LA becoming denatured.

In addition to changes in secondary structure, the antibody binding may also be affected by the formation of thermally-induced aggregates. Both α -LA and β -Lg form large aggregates many millions of daltons in molecular weight of following heating of the purified

TABLE 2. Model heat treatments employed to simulate commercial dairy processes

Heat treatment		Regime
Pasteurization	Batch heating	65°C/30 min
	Short time heating	75°C/20 s
	High temperature heating	85°C/15 s
UHT treatment		140°C/4 s
Sterilization		115°C/15 min

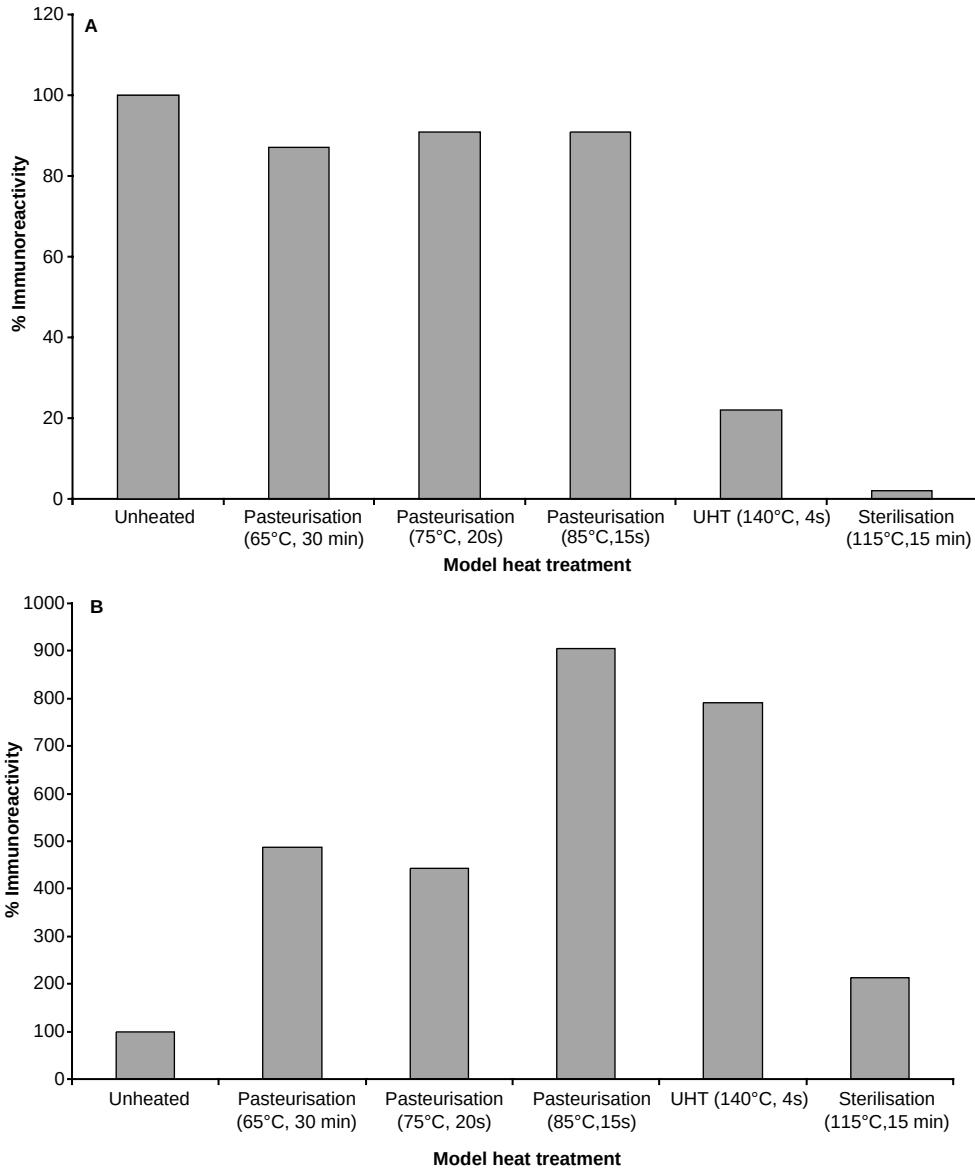


FIG. 5. Effect of model heating regimes that mimic technological processes on the immunoreactivity of α -lactalbumin (A), and β -lactoglobulin variant B (B), as determined by inhibition ELISA. Proteins (0.2 mg ml^{-1} in phosphate buffered saline) were heated according to the regimes in Table 2 prior to analysis by the appropriate immunoassay.

proteins (Clark, 1988; Ikeda & Morris, 2002). The effect of heat-treatment on aggregation of these whey proteins was not investigated in this study. However, the major oligomer present after heating β -Lg to 67.5°C for over 100 min is thought to be a disulphide-linked dimer which goes on to form larger aggregates held together by hydrophobic interactions (Carrota *et al.*, 2001). In general such aggregates might be expected to reduce the availability of antibody recognition sites by reducing their accessibility. Thus, the reduction in

TABLE 3. Immunoreactivity of the whey proteins α -lactalbumin and β -lactoglobulin in thermally-treated milks. The levels of immunoreactive whey proteins were determined using inhibition ELISAs for α -lactalbumin and β -lactoglobulin variant B. On the assumption that the absolute levels of whey proteins are similar in all milk samples, the alterations in immunoreactivity of the whey proteins in treated milks was calculated as a percentage relative to the amounts of immunoreactive protein present in the raw milk samples. Data are quoted $\pm 2\sigma$ and are mean values from several determinations (see Table 2)

Milk treatment	Immunoreactive whey protein (mg in 1 ml of milk)		% Immunoreactivity	
	α -Lactalbumin	β -Lactoglobulin	α -Lactalbumin	β -Lactoglobulin
Raw	1.63 $\pm$ 0.41	1.05 $\pm$ 0.24	100	100
Pasteurized	1.59 $\pm$ 0.50	14.71 $\pm$ 3.65	100 $\pm$ 19 <i>n</i> = 7	1401 $\pm$ 312 <i>n</i> = 7
UHT	0.38 $\pm$ 0.04	6.04 $\pm$ 1.82	18 $\pm$ 2 <i>n</i> = 9	563 $\pm$ 138 <i>n</i> = 9
Sterilized	0.016 $\pm$ 0.005	1.65 $\pm$ 0.50	1.0 $\pm$ 0.1 <i>n</i> = 5	141 $\pm$ 28 <i>n</i> = 5

immunoreactivity observed on heating α -LA may be in part due to protein aggregation as well as unfolding. However, the effect of aggregation on immunoreactivity can be complex and previous studies have shown that antibody binding to mobile sites on proteins may be unaltered by heat-induced aggregation (Huang *et al.*, 1998).

In contrast the antibody preparations raised to β -Lg variants A and B both preferentially recognised the thermally denatured protein, the increases immunoreactivity again coinciding with the alterations in secondary structure associated with thermal denaturation. There is strong evidence that β -Lg heated to 70–90°C forms a partially unfolded intermediate, termed by some workers as a “molten globule” (Casal *et al.*, 1988). Such entities retain a high degree of native secondary structure, but exhibit a far greater degree of flexibility than is found in the native proteins. It seems likely that the antisera to both variants preferentially may recognize such a partially folded state of β -Lg, despite having been raised to the native protein. This may be a result of the emulsification of β -Lg in oil prior to immunization, as β -Lg has been found to undergo a small degree of unfolding when adsorbed to the oil:water interface (Husband *et al.*, 2000).

All the anti-whey protein antibody preparations described in this study were able to detect similar thermally-induced alterations in the whey proteins in whole milk. Thus, the anti- α -LA antiserum, whilst not able to discriminate between raw and pasteurized milk, was effective at discriminating between raw and more severely heated milks. This reactivity was complimented by the anti- β -Lg variant B antiserum, which was able to discriminate between more severely treated milks, as well as between raw and pasteurised milk. Hence, by using a combination of antibody preparations, it is possible to discriminate between all the milk samples, whatever their thermal treatment and obtain information about the conformational state of proteins in foods *per se* which are difficult to analyse using spectroscopic methods due to their opacity. We have also demonstrated the principle that immunoassays can be used as effective methods to monitor thermal processing of foods with out recourse to sophisticated biophysical methods not widely available in the food industry for quality control purposes.

ACKNOWLEDGEMENTS

This work was funded by the European Union, CT 94-1010 and the Research Support CEJ: J19/98:223300004 of the Ministry of Education of the Czech Republic.

REFERENCES

- AYMARD, P., DURAND, D. & NICOLAI, T. (1996) The effect of temperature and ionic strength on the dimerisation of β -lactoglobulin. *International Journal of Biological Macromolecules*, **19**, 213–221.
- BAUM, J., DOBSON, C. M., EVANS, P. A. & HANLEY, C. (1989) Characterization of a partly folded protein by NMR methods: studies on the molten globule state of guinea-pig α -lactalbumin. *Biochemistry*, **28**, 7–13.
- BELL, A., MCKENZIE, H. A. & SHAW, D. C. (1981) Bovine β -lactoglobulin E,F and G of Bali (Bateng) cattle *Bos (Bibos) javanicus*. *Australian Journal of Biological Science*, **34**, 133–147.
- BREW, K. & GROBLER, J. A. (1992) α -Lactalbumin., in *Advances in Dairy Chemistry Vol 1, Proteins* (Fox, P. F., Ed.). Elsevier Applied Science, New York, pp. 191–229.
- BROWNLOW, S., MORAIS-CABRAL, J. H., COOPER, R., FLOWER, D. R., YEWDALE, S. J., POLIKARPOV, I., NORTH, A. C. T. & SAWYER, L. (1997) Bovine β -lactoglobulin at 1.8 Å resolution – still an enigmatic lipocalin. *Structure*, **5**, 481–495.
- CARROTTA, R., BAUER, R., WANINGE, R. & RISCHER, C. (2001) Conformational characterisation of oligomeric intermediates and aggregates in β -lactoglobulin heat aggregation. *Protein Science*, **10**, 1312–1318.
- CASAL, H. L., KÖLER, U. & MANTSCH, H. H. (1988) Structural and conformational changes of β -lactoglobulin B: an infrared spectroscopic study of the effects of pH and temperature. *Biochimica et Biophysica Acta*, **957**, 11–20.
- CHAKRABORTY, S., ITTAH, V., BAI, P., LUO, L., HAAS, E. & PENG, Z. (2001) Structure and dynamics of the α -lactalbumin molten globule: fluorescence studies using proteins containing a single tryptophan residue. *Biochemistry*, **40**, 7228–7238.
- CHO, Y., BATT, C. A. & SAWYER, L. (1994) Probing the retinol-binding site of bovine β -lactoglobulin. *Journal of Biological Chemistry*, **269**, 11102–11107.
- CLARK, A. H. (1998) Gelation of globular proteins., in *Functional Properties of Food Macromolecules* (MITCHELL, J. R., Ed.). London Elsevier Applied Science, pp. 77–142.
- CREAMER, L. K. (1995) Effect of sodium dodecyl sulfate and palmitic acid on the equilibrium unfolding of on bovine β -lactoglobulin. *Biochemistry*, **34**, 7170–7176.
- HUANG, L., MILLS, E. N. C., CARTER, J. M. & MORGAN, M. R. A. (1998) Analysis of thermal stability of soya globulins using monoclonal antibodies. *Biochimica et Biophysica Acta*, **1338**, 215–226.
- HUSBAND, F. A., GARROOD, M. J., MACKIE, A. R., BURNETT, G. & WILDE, P. J. (2000) Adsorbed protein secondary and tertiary structure by circular dichroism and infrared spectroscopy with refractive matched emulsions. *Journal of Agricultural and Food Chemistry*, **49**, 859–666.
- IDF (1991) Heat-treatment of milk – determination of lactulose content, Provisional International Standard 147: IDF: Brussels, Belgium.
- IKEDA, S. & MORRIS, V. J. (2002) Fine-stranded and particulate aggregates of heat-denatured whey proteins visualised by atomic force microscopy. *Biomacromolecules*, **3**, 382–389.
- JEANSON, S., DUPONT, D., GRATARD, N. & ROLET-RÉPÉCAUD, O. (1999) Characterisation of the heat treatment undergone by milk using two inhibition ELISAs for quantification of native and heat denatured α -lactalbumin. *Journal of Agricultural and Food Chemistry*, **47**, 2249–2254.
- KUZMANOFF, K. M. & BEATTIE, C. W. (1991) Isolation of monoclonal antibodies monospecific for bovine (β -lactoglobulin. *Journal of Dairy Science*, **74**, 3731–3740.
- LEONIL, J., MOLLE, D., FAUQUANT, J., MAUBOIS, J. L., PEARCE, R. J. & BOUHALLAB, S. (1997) Characterisation by ionisation mass spectrometry of lactosyl (β -lactoglobulin conjugates formed during heat treatment of milk and whey and identification of one lactose-binding site. *Journal of Dairy Science*, **80**, 2270–2281.
- MANDERSON, G. A., HARDMAN, M. J. & CREAMER, L. K. (1999) Effect of heat treatment on bovine β -lactoglobulin A, B, and C explored using thiol availability and fluorescence. *Journal of Agricultural and Food Chemistry*, **47**, 3617–3627.
- MCKENZIE, H. A., NORTON, R. S. & SAWYER, W. H. (1971) Heat induced interaction of β -lactoglobulin and κ -casein. *Journal of Dairy Science*, **38**, 343–351.
- MONGET, D. & LAVIOLETTE, P. (1978) Alkaline phosphatase and peroxidase microtests for control of pasteurisation of cows milk. *Lait*, **58**, 595–605.
- MOTTAR, J., BASSIER, A., JONIAU, M. & BAERT, J. (1989) Effect of heat-induced association of whey proteins and casein micelles on yoghurt texture. *Journal of Dairy Science*, **72**, 2247–2256.
- NEGRONI, L., BERNARD, H., CLEMENT, G., CHATEL, J. M., BRUNE, P., FROBERT, Y., WAL, J. M. & GRASSI, J. (1998) Two-site enzyme immunometric assays for determination of native and denatured beta-lactoglobulin. *Journal of Immunological Methods*, **220**, 25–37.
- QIN, B. Y., BEWLEY, M. C., CREAMER, L. K., BAKER, E. N. & JAMESON, G. B. (1999) Structural and functional differences of variants A and B of β -lactoglobulin. *Protein Science*, **8**, 1–9.
- SMITS, P. & VAN BROUWERSHAVEN, J. H. (1980) Heat-induced association of β -lactoglobulin and casein micelles. *Journal of Dairy Research*, **47**, 313–325.

- VAN REGENMORTEL, M. H. V. (1992) Molecular dissection of protein antigens., in *Structure of Antigens* (VAN REGENMORTEL, M. H. V., Ed.), vol. 1. Boca Raton, FL, CRC Press Inc, pp. 1–28.
- WALSTRA, P. & JENNES, R. (1984) Protein composition of milk., in *Dairy Chemistry and Physics* (WALSTRA, P. & JENNES, R., Eds). John Wiley & Sons, Inc., New York, pp. 107–117.