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RESEARCH ARTICLE

TRACEABILITY INDICATORS FOR HEAT TREATMENTS OF MILK

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ABSTRACT

Because of its high nutritional value, milk is an excellent medium for microbiological growth. Consequently, fresh milk necessitates a heat treatment in order to guarantee a safe and shelf-stable product. To overcome processing of the product as well as to control if heating was adequate from a safety point of view, criteria need to be defined. In order to define such criteria, the impact of a thermal process on milk has to be known.

The changes in solubility of β-LG solutions as an indicator for milk pasteurization and the color development measured by changes in absorbance at 420 nm in milk-resembling model systems at pH 7.5 as indicator for sterilization process were used in this study to quantitatively describe the heat induced changes in model systems. The extent of changes measured by the decrease in solubility and increase in absorbance could be described by a first-order fractional conversion model, which allowed calculation of activation energy.

KEY WORDS: traceability, heating, milk protein, Maillard reaction, kinetic

INTRODUCTION

For neutral pH foods at room temperature, the various microorganisms have to be reduced to negligible numbers to give storage stability and to assure safety criteria. Initially, heat pasteurization was very valuable and this was complemented by sterilized tin-plated steel cans of concentrated milk, meat, fruit and vegetables (Considine et al., 2007).

Because of its high nutritional value, milk is an excellent medium for microbiological growth. Consequently, fresh milk necessitates a heat treatment in order to guarantee a safe and shelf-stable product. Numerous reactions may then occur, depending on the duration and extent of heating, which cause significant changes in milk constituents, some of which are undesirable, particularly those affecting the solubility and nutritional value of proteins (Morales, 2000). To overcome processing of the product as well as to control if heating was adequate from a safety point of view, criteria need to be defined. Such criteria not only must guarantee the correctness of a heat treatment, but can also be applied for quality management at all steps in the production process. Moreover, these criteria must result in products on the market that comply with their labeling in terms of processing (thermization, pasteurization, sterilization). In order to define such criteria, the

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impact of a thermal process on milk has to be known.

There are two essential ways to account for the effects of the heat treatment: to select pertinent chemical indicators sensitive to a given range of heat treatments, or to globally evaluate the heat effect using a physicochemical approach.

Pellegrino et al. (1995) defined two ways for distinguish heat treatment of milk:

- whey proteins;
- hydroxymethylfurfural (HMF);
- tyamine;
- lysine;
- lactuloze;
- epilactoze;
- furozine;
- N -6-methyladenozine;
- N-ε-carboximethyl-lisine (CML);
- colour changes;
- flavor compounds.

Heat treatment affects the sensory, biophysical and nutritional properties of milk. The main events occurring upon heating are, successively, protein denaturation, Maillard reaction and lactose isomerization.

Whey proteins constitute aproximatively 20% of milk proteins and are significant for their variable sensitivity to heat so they can act as suitable indicators for monitoring the heat treatment of milk. β-lactoglobulin (β-LG) is the main protein in whey, constituting about 50% of the total whey proteins in bovine milk.

1. *denaturation* of heat-sensitive compounds such as enzymes (alkaline phosphatase, peroxidase) and proteins (β-lactoglobulin), and
2. formation of *specific indicators* such as lactulose, furosine and hydroxymethylfurfural, correlated with each other.

The European Regulation No. 2597/97 is defined some intrinsic indicators of heat treaments of milk:

The non-enzymatic browning or Maillard reaction is of major importance in food preparation. (Chobert et. al., 2006). The Maillard reaction is of prime importance to both food scientists and food processors, as it affects the quality of processed food products, in particular the sensory attributes, like colour, flavour and taste (Martins and van Boekel, 2005).

The objective of this study was to follow the heat induced changes in solubility of β-LG solutions as an indicator for milk pasteurization and the color development measured by changes in absorbance at 420 nm in milk-resembling model systems at pH 7.5 as indicator for sterilization process.

MATERIALS AND METHODS

Preparation of reaction mixtures

Milk-resembling model systems casein: whey proteins concentrate: lactose ratio:1: 0.2456: 1.579 (CN/WPC/L) were prepared in 100 mL 0.7 M phosphate buffer at pH 7.5. All solvents and chemical reagents were of analytical grade.

Isothermal treatment of solutions

β-LG solutions (110 μl of 2.5 mg/mL in 0.02 *M* Tris-HCl buffer, pH 7.5) were heated in 1.5 ml flexible centrifuge tubes (Eppendorf) in a thermostated water bath at constant temperatures between 70-85°C for 1-40 minutes.

One mL of proteins/sugar solutions were filled in the screw-capped glass tubes. The thermal treatment experiments were performed in a thermostatic oil bath at various constant temperatures (90-130°C) for preset time intervals (0 - 40 minutes).

After thermal treatment, samples were immediately transferred to ice water to prevent further denaturation. Analysis of the heat-induced changes was always performed exactly 2 min after thermal treatment.

Solubility

Diluted samples of (un)treated β-LG solutions were centrifuged for 15 min (Eppendorf 201 centrifuge) at 19900 g and 4°C. Protein concentration in the supernatant was determined using Sigma Procedure n.° TPRO-562. Bovine serum albumin was used as a standard. All samples

were assayed in duplicate. Solubility was expressed as the percentage of protein content in the supernatant compared to the total protein content of the untreated sample.

Maillard reactions

The procedure was described by Nakai *et al.*, (1964) as follow: to 0.5 mL (un)heated protein solutions 0.5 mL butylamine was added. The solutions were hold in water bath at 65^0 C/5 minutes, two drops of 5% $NaBH_4$ was added and then the mixture was allowed to stand again 5 minutes at 65^0C. After this period 25 mL of diluting agent (3 g of ethylenediaminetetraacetic acid – EDTA and 3 g of sodium lauryl sulphate - SLS in 500 ml water) was added. Five drops of HCl was used to neutralize the solution and then filter through No. 42 Whatman paper. One drop of 30% NaOH was added and optical density at 280 nm was measured after 15 minutes using water as blank. Absorbance was determined in a UV–VIS GBC Cintra 202 spectrophotometer.

Kinetic data analysis

Changes in solubility and absorbance as a function of heating time could be described by a fractional conversion model (a modified first order kinetic model) (**equation 1)**:

$$X_t = X_\infty + (X_\infty - X_i)\exp(-kt) \quad \textbf{(1)}$$

with X_∞ the equilibrium value of the response value X at infinite heating time and X_i the response value of the native proteins at time t = 0.

The temperature dependence of the rate constant, k (min^{-1}) could be described by the Arrhenius **equation (2)**:

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

with T and T_{ref} the absolute and the reference temperature (K) respectively, k_{ref} the rate constant at T_{ref}, E_a the activation energy ($kJmol^{-1}$) and R the universal gas constant (8.314 $Jmol^{-1}K^{-1}$). Kinetic parameters were estimated by nonlinear regression analysis (*SAS, 1999-2001*).

RESULTS AND DISCUSSION

Heat-induced changes in the solubility of β-LG solutions

Heat treatment of milk follows two main objectives, which are to ensure a good microbiological quality and to allow long storage. Nowadays, two main heat treatments are used for commercial milk, namely, pasteurization implicating a short storage period (less than 21 days) at maximum 4^0C, and UHT treatment allowing much longer storage time (2–3 months) at room temperature. However, various systems and technologies associated with different time–temperature profiles are now developed to obtain pasteurized and UHT milk, and the effect on the nutritional and sensory quality of commercial milk samples may vary substantially depending on the process (Birlouez-Aragon et al., 2002).

There are few quantitative data in literature regarding the thermal indicators of milk and milk products, but it is very difficult to compare and use these informations as traceability indicators of heat-treated milk, because of the variability of analytical methods, composition of milk, kinetics, etc. The lack of official indicators of the heat treatment and the high cost of the standardized HPLC techniques allowing quantification of some referenced indicators may explain the lack of control of the nutritional value of heat-processed milk.

Solubility, however, is an important characteristic for functional application of proteins in food systems, not only per se but also as a prerequisite for derived functional properties like emulsification, foaming, and gelation (De Witt and Klarenbeek, 1984). The commonly used heat treatments such as pasteurization and sterilization always affect the structure and properties of whey proteins, either reversibly or irreversibly.

The quantitative analysis of β-LG as pasteurization marker was achieved by using the Sigma Procedure n.° TPRO-562 method to measure the loss in solubility after thermal treatment. A kinetic study on the structural heat-induced changes in β-LG should lead to a better understanding of the relationship between heat treatment and its effect on the functional properties of β-LG, with the

perspective of new applications of whey proteins in foods (de la Fuente et al., 2002*)*. Additionally, a kinetic model for the thermal denaturation of β-LG is important for optimising heat treatment of milk products so that the desired functional properties are achieved.

Kinetics plots of the solubility of β-LG heated in 0.02 *M* Tris-HCl buffer at pH 7.5 are given in **Figure 1**.

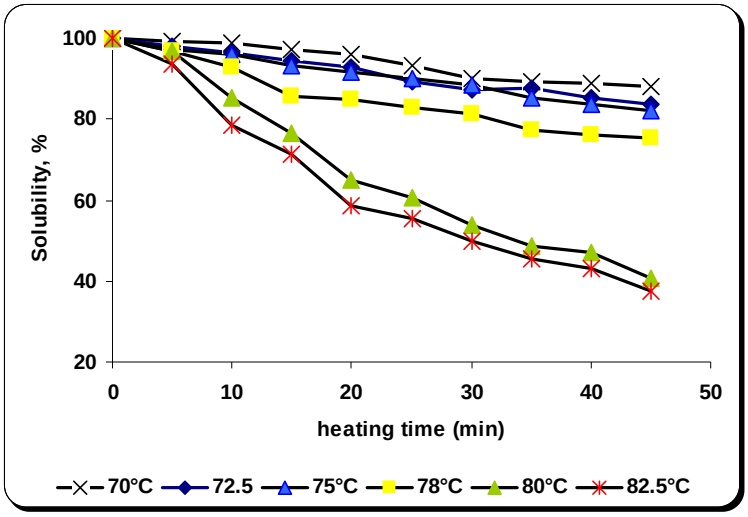

Figure 1. Time dependent heat-induced structural changes in β-LG solubility (2.5 mg/ml, pH 7.5)

It can be seen that heating at a temperature between 70 and 75°C results in a minimal loss β-LG in solution, a decrease in solubility of only 10-20% compared with the native protein was observed after 45 min of heating, probably because at lower temperature, the intramolecular interchange reactions are favoured. These results are in good agreement with the data concerning heat-induced changes in turbidity and surface hydrophobicity β-LG solutions as a function of temperature (Sava et. al., 2005). Partial protein denaturation and its solubility coincide to some extent and good solubility of β-LG after thermal treatment under neutral conditions and at low ionic strength is theoretically expected (De Witt and Klarenbeek, 1984) and actually observed.

When too many hydrophobic sites are exposed, due to thermal treatment, the hydrophobic interactions are enhanced, usually leading to a decrease in solubility. However, thermal treatment above 80°C results in protein aggregation, with a decrease in solubility of 60%. This observation indicates that thermal denaturation of β-LG as measured by the changes in solubility involves two steps: an unfolding step (70-75°C) and an aggregation step (78-82.5°C), that mostly follows unfolding, leading to a major decrease in solubility.

Heat-induced changes in the absorbance of milk-resembling model systems

From a general point of view, thermal treatment improves food safety, sensory qualities and shelf life of dairy products. However, heat treatment may adversely affect product quality, safety and nutritional value by means of oxidative damage to food proteins. Several chemical markers are commonly used for assessing these modifications. One of the most important targets for modification in milk products is the protein entity. In particular, the well known Maillard reaction, occurring during food manufacture and storage, is responsible for decreasing protein digestibility and nutritional supply. In its early stage the Maillard reaction can be evaluated by measuring the furosine content using HPLC methodologies (Resmini et al., 1990) or by measuring milk protein glycation by mass spectrometry techniques (Fenaille et al., 2006). In the advanced stage of the Maillard reaction, advanced glycation end-products such as N^{ε}-(carboxymethyl)lysine are formed.

The mechanism and the profile of products of Maillard reaction are very complicated, not much

is yet known about the chemistry of the browning components and certainly not in milk. Therefore, researchers commonly use model systems to limit the scope.

Kinetics plots of the intermediary Maillard reaction stage measured by absorbance of milk-resembling model systems in 0.7 M phosphate buffer at pH 7.5 are given in **Figure 2**. The plots show that depending on the temperature-time combination, the absorbance gradually increase, especially in higher temperature range. At 90^0 C, the increase in absorbance seems to follow a linear tendency (R^2 = 96%). As it can be seen, the extent of reaction is more advanced as the temperature-time combination increases. Taking into consideration that proteins strongly absorb at 280 nm, it should be mentioned that the given absorbance values were calculated abstracting values at time 0 (native solutions).

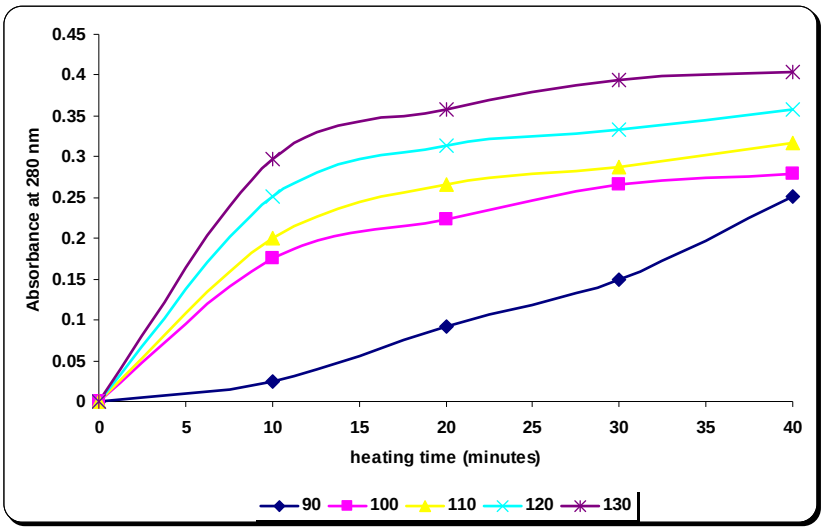

Figure 2. Time dependent changes in milk resembling model systems heated in 0.7 M phosphate buffer at pH 7.5 at different temperatures

The maximum extent of browning was reached at 130^0C after 40 minutes of holding, being 1.7 times higher than the initial value. The higher values recorded during the intermediate stages may be due to the high reactivity of the di - and polycarbonyl compounds generated during these stages (Ajandouz et al., 2008).

Kinetics analysis

Kinetic studies are an excellent means of predicting and managing food browning processes, as mentioned by van Boekel (2001). The activation energy had been the important and widely used parameters for predicting the effect of temperature on any specific reaction.

In this study, the first-order fractional conversion model could be applied to describe the heat-induced changes in solubility of β-LG solutions and absorbance of milk protein/lactose model systems.

The kinetics describing the heat induced-changes in solubility of β-LG solutions showed a break in the Arrhenius plot around 80°C (**Figure 3**), resulting in a clear distinction of E_a values in the two temperature ranges studied (**table 1**).

This is possible due to the complexity of the irreversible thermal denaturation process of β-LG, involving a number of successive reaction steps. A possible model for the thermal behaviour of β-LG in buffer involves three steps as reported by Roefs and Kruif (1994). The early step is the dissociation of the dimer to monomer favoured at neutral pH, followed by a heat-induced unfolding step with the exposure of the free reactive thiol group and hydrophobic residues. The last step involves the aggregation of the molecules, due to

sulphydryl/disulfide interchange and non-covalent reactions.

Table 1. Kinetic parameters k and E_a of the first order fractional conversion model describing heat-induced changes in solubility of β-LG solutions and browning of milk model systems

Pasteurization marker		**Sterilization markers**	
Temperature (°C)	**k (min^{-1})**	**Temperature (°C)**	**k (min^{-1})**
70	0.000844±0.007[a]	90	-
72.5	0.00274±0.005	100	0.0956±0.013
75	0.0097±0.013	110	0.1065±0.016
78	0.027039±0.02	120	0.1219±0.013
80	0.029047±0.018	130	0.1290±0.0085
82.5	0.03907±0.024		
85	0.043197±0.03		
E_a ($kJ.mol^{-1}$)	[1]439.26±24.7	**E_a ($kJ.mol^{-1}$)**	12.95±1.23
	[2]76.012±10.07		

[a]: values ± standard errors of regression;
[1] 70-78°C; [2] 78-85°C.

The Arrhenius plot becomes nonlinear above the transition temperature, which may be caused by the heat-induced aggregation of the protein and expressed in the second part of the curve. A break in the linear Arrhenius plots around 85°C was also reported in literature (Dannenberg and Kessler, 1988, Anema and McKenna, 1996, Claeys, 2003, Sava et; al., 2005). This unusual behaviour could be a consequence of different rate-determining steps involving the participation of two consecutive reactions in the denaturation process, characterized by two different E_a values, as explained by Anema and McKenna (1996).

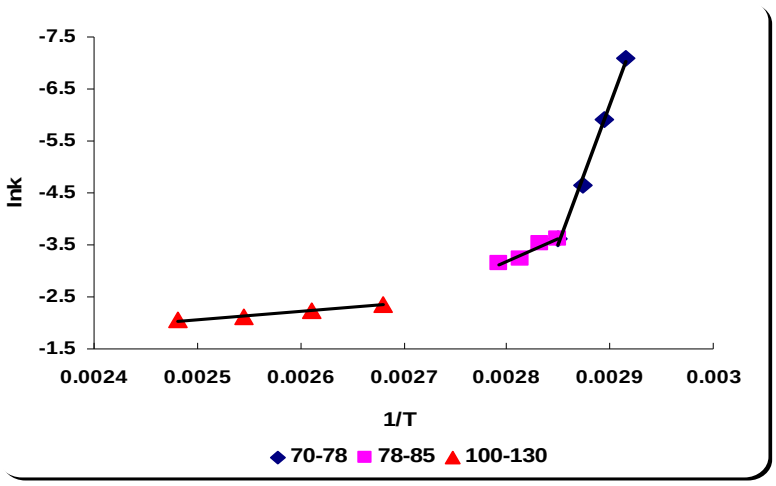

Figure 3. The temperature dependence of the rate constant (k, min^{-1}) describing changes in β-LG solubility in 0.02 *M* Tris-HCl buffer and browning at pH 7.5

As for the heat-induced changes in browning measured as absorbance at 280 nm, the temperature dependence of the *k* values in the temperature range of 100 to 130°C (**Figure 3**) could be described also by the Arrhenius equation, resulting in an activation energy of 12.95±1.23 $kJ.mol^{-1}$ ($r^2 = 0.98$).

Ajandouz et al., (2008) also observed an increase in non-enzymatic browning, measured as changes in absorbance at 420 nm for a glucose-casein system. They reported activation energy of 128 $kJ.mol^{-1}$ at pH 8.0 and 107 $kJ.mol^{-1}$ at pH 9.7. Our Ea value was somewhat lower than those obtained on other reducing sugar–protein model systems, and the factors such as pH and water activity may possibly have contributed to this difference, although it is difficult to allot a specific score to each factor. We reported elsewhere activation energy of 21.5±1.6 $kJ.mol^{-1}$ in phosphate buffer and a lower value (16.5±1.65 $kJ.mol^{-1}$) in acetate buffer at pH 7.5. These heat-induced changes were quantitatively described as changes in Br index for a milk-resembling model system. It seems that the system doesn't require too much energy cost to initiate the non-enzymatic browning reactions.

CONCLUSIONS

Heat treatment of β-LG at neutral pH causes the dimeric native protein to dissociate, partially unfold, denature and aggregate; the rates and pathways were dependent on the temperature-time combination. Two major aggregation features, or possibly mechanisms, are related to hydrophobic association and disulfide-bond interchange reactions.

The kinetics was monitored by following the solubility of β-LG when heated between 70 and 85 °C at pH 7.5 for various times. A first-order fractional conversion model was applied to describe the heat-induced changes in the solubility of β-LG. These showed a temperature dependence of the k values (rate constants) with a break in Arrhenius plots around 78^0C.

Sava et al. (2005) suggested different rate-determining steps involving the participation of two consecutive reactions in the denaturation process. At lower temperatures (70–78 °C), the rate-determining step is unfolding of the molecules, whereas, at higher temperatures (78–82.5 °C), the aggregation process involving unfolded molecules becomes rate determining. More recently, Considine et al. (2007) proposed calling these steps Stage I T (native protein) and Stage II T (heat-denatured protein), as defined by the absence of native disulfide bonding after the heat treatment. For simplicity, Stage I T signifies at least 90% native β-LG structure whereas the β-LG in Stage II T may include up to 20% native protein.

The rate of advanced Maillard products formed in milk protein/lactose model systems measured as changes in the absorbance at 420 nm was dependent on temperature-time combination, with a very low activation energy compared with the data from literature. The study found that the rate of color development in Maillard reaction followed a fractional conversion model.

The results showed in this study suggested that increasing temperature leaded to an increase of the reaction rate, as this will reduce the energy difference between the initial state (reactants) and the activated complex.

Further studies are needed to provide insights into the mechanism of protein denaturation and Maillard reaction in milk. These specific and complex issues are some of the main objectives of our Research Project PN-II-ID-PCE-2008-2, Idea, ID 517 – *Research resulting in analytical systems for Romanian milk and dairy products traceability in order to comply with European quality and safety criteria*.

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