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# DENATURATION IMPACT IN SUSCEPTIBILITY OF BETA-LACTOGLOBULIN TO ENZYMATIC HYDROLYSIS: A KINETIC STUDY

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The aim of this study was to establish a kinetic model to describe the effect of heat processing on the susceptibility of β-lactoglobulin to proteolysis. The kinetic study was performed in the temperature range of 62.5-78°C on the susceptibility of β-lactoglobulin to enzymatic hydrolysis by trypsin and chymotrypsin at 37°C and pH 7 and 7.5. Heat treatment results in an increase in degree of hydrolysis after 10 min of hydrolysis, as monitored by pH-stat technique. For the hydrolysis experiments at pH 7, the values for activation energy were significantly higher for the susceptibility of β-lactoglobulin to trypsin hydrolysis compared with chymotrypsin. In contrast, for the hydrolysis experiments at pH 7.5, β-LG seems to be more heat-sensitive for chymotrypsin hydrolysis, confirming that the dimer dissociates when the pH is adjusted at 7.5, with the exposure of hydrophobic residues.

## INTRODUCTION

β-lactoglobulin (β-LG) is the main protein in whey, constituting about 50% of the total whey proteins in bovine milk. At room temperature and physiological pH of milk, β-LG exists mainly as a non-covalently linked bi-unit, stabilized by hydrogen bonds.[1] The compact globular structure of β-LG contains a β-barrel, consisting of eight antiparallel β-strands forming a central hydrophobic core.[2] The β-barrel is stabilized by an intramolecular disulphide bond between residues $Cys^{106}$-$Cys^{119}$ and $Cys^{66}$-$Cys^{160}$. Many authors consider β-LG responsible for the allergenic reaction because of its absence in human milk.[3] Native protein is not hydrolyzed easily by pepsin, but is susceptible for chymotrypsin and trypsin hydrolysis, under nondenaturing conditions.[4] The poor digestibility of β-LG is considered to be the reason for its allergenicity.[5] The relative resistance to proteolysis is generally explained by the compact tertiary structure of the protein that protects most of the enzyme susceptible peptide bonds. Physical and/or chemical denaturation of β-LG generally leads to a higher rate of hydrolysis by various enzymes.[5-11] After denaturation, the β-LG conforms may represent the ideal substrates for the proteases, because ample regions of the hydrophobic protein core are unfolded and accessible for the enzyme, contrarily to what happens in the native form or in the aggregated products of extensive thermal denaturation.[12] It is possible, also, that the heat-induced changes to hide some susceptible bonds for the enzymes that may be relevant for its allergenic reactions.

Susceptibility to enzymes hydrolysis is used as an index of flexibility since partial unfolding of protein molecules generally results in increased hydrolysis rate.[4] Since β-LG is heat sensitive, thermal treatment may affect its digestibility characteristics and it biological availability. The aim of this study was to follow the heat-induced changes in the susceptibility of β-LG to enzymatic hydrolysis at neutral pH for the heat-treated protein. The degree of hydrolysis after 10 minutes of reaction (further referred to as DH10) obtained with different enzymes was used as a parameter to quantitatively describe the effect of heating on the

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susceptibility of β-LG to proteolysis, at high enzyme:protein ratio.

## MATERIALS AND METHODS

β-LG (90% pure), trypsin (EC 3.4.21.4, type IX-S from bovine pancreas, 15.450 units/mg protein) and α-chymotrypsin (3.4.21.1, type II from bovine pancreas, 51 units/mg protein, $E_{280}^{1\%}$) were purchased from Sigma Chemical Co (USA). All solvents and chemical reagents were of analytical grade.

### Heat treatments

Aqueous solutions (3.8 ml of 2.5 mg/ml β-LG, pH 7) were filled in the plastic tubes (1 cm diameter). The thermal treatment experiments were performed in a thermostatic water bath at various constant temperatures (62.5-78°C) for preset time intervals (0 - 30 minutes). After thermal treatment, the tubes were immediately cooled in ice water to prevent further denaturation. The changes in susceptibility of β-LG to enzymatic hydrolysis were measured exactly in 2 min after thermal treatment.

### Enzymatic Hydrolysis of β-LG solutions

The susceptibility of β-LG to enzymatic hydrolysis was determined in aqueous dilutions after heat treatment, by adding 1.5 mL enzymes solution to 15 mL of diluted heat-treated sample (0.625 mg/mL w/v, E:S ratio 1:10). Trypsin and chymotrypsin were chosen because of their high specificity and complementary mechanisms of action.[13]

Quantification of DH10 by the pH-stat method was carried out using the data obtained with a 718 Titrino (Metrohm, Basel, Switzerland). For the calibration of the pH-stat method, pH was 7, 7.5 or 8, while the pH was kept at 7 and 7.5 during the actual enzymatic protein hydrolysis experiments. The pH was kept constant during the enzymatic hydrolysis by automatic dosage of the base (0.01N NaOH). The number of peptide bonds cleaved can be estimated from the amount of base required to maintain a constant pH during the enzymatic reactions. The degree of hydrolysis (DH) was calculated based on equation 1:[14]

$$DH(\%) = \frac{B \cdot N_b}{\alpha \cdot M \cdot h_{tot}} \cdot 100 \qquad (1)$$

with B the base consumption (mL) during hydrolysis at 37°C, $N_b$ the normality of the base (meq/mL), α the degree of dissociation of α-$NH_2$ groups, M the mass of the protein in experiment (mg), and $h_{tot}$ the total number of the peptide bonds in the protein substrate (meq/mg).

The degree of dissociation (α) for the α-$NH_2$ groups was estimated with equation 2:

$$\alpha = \frac{10^{(pH-pK)}}{1+10^{(pH-pK)}} \qquad (2)$$

with pK the average dissociation value for the α-amino acids liberated during enzymatic hydrolysis.

The pK values were estimated by determining the increase in free amino groups in the resulting hydrolysates from pH-stat with *o*-phtaldialdehyde (OPA) method.[15] The samples were drawn during the pH-controlled hydrolysis after various predefined hydrolysis time at different pH values (7, 7.5 and 8). The OPA assay was carried out by the addition of 150 μL of hydrolysates to 3 mL of OPA reagent. The solution was briefly mixed by inversion and the absorption was measured at 340 nm after 2.5 min of incubation at room temperature. A standard curve was prepared with L-leucine (0–1.5 mM) and used to calculate the L-Leucin equivalents ($h_{Leu}$). The amount of sodium hydroxide consumed during hydrolysis was converted to hydrolysis equivalents ($h_{pH\text{-}stat}$). After correlating hydrolysis equivalents ($h_{pH\text{-}stat}$) and $h_{Leu}$, straight lines of slope b were obtained. The pK values were calculated using the equation 3, in which $pH_2 > pH_1$, and $b_2$ and $b_1$ are the slopes of the straight line at the respective pH value:[14]

$$pK = pH_2 + \log(b_1 - b_2) - \log\left(10^{pH_2 - pH_1} \cdot b_2 - b_1\right) \qquad (3)$$

### Kinetic Data Analysis

The fractional conversion model (a modified first order kinetic model) was used to define the heat-induced changes on the susceptibility of β-LG to enzymatic hydrolysis. In this model, the changes in DH10 as a function of heating time are described by equation 4:[16]

$$DH10_t = DH10_\infty + (DH10_\infty - DH10_i)\exp(-kt) \quad (4)$$

with $DH10_\infty$ is the equilibrium value for DH10 at infinite heating time (the value after which longer heating time does not result in changes in the DH) and $DH10_i$ is the degree of hydrolysis of the samples at time 0 of thermal treatment.

The temperature dependence of the rate constant, k ($min^{-1}$) was described by the Arrhenius equation (5):

$$k = k_{ref} \exp\left(-\frac{E_a}{R}\left(\frac{1}{T} - \frac{1}{T_{ref}}\right)\right) \quad (5)$$

with T and $T_{ref}$ the absolute temperature (K) and the reference temperature (K), respectively; $k_{ref}$ the rate constant at $T_{ref}$, $E_a$ the activation energy ($kJmol^{-1}$), R the universal gas constant (8.314 $Jmol^{-1}K^{-1}$). Kinetic parameters were estimated by nonlinear regression analysis.[17]

## RESULTS AND DISCUSSION

**pH-stat calibration.** The calibration of the pH-stat method was performed separately for both enzymes. A linear relationship between base consumption and concentration of free amino-acids groups was obtained for each pH value (Fig. 1).

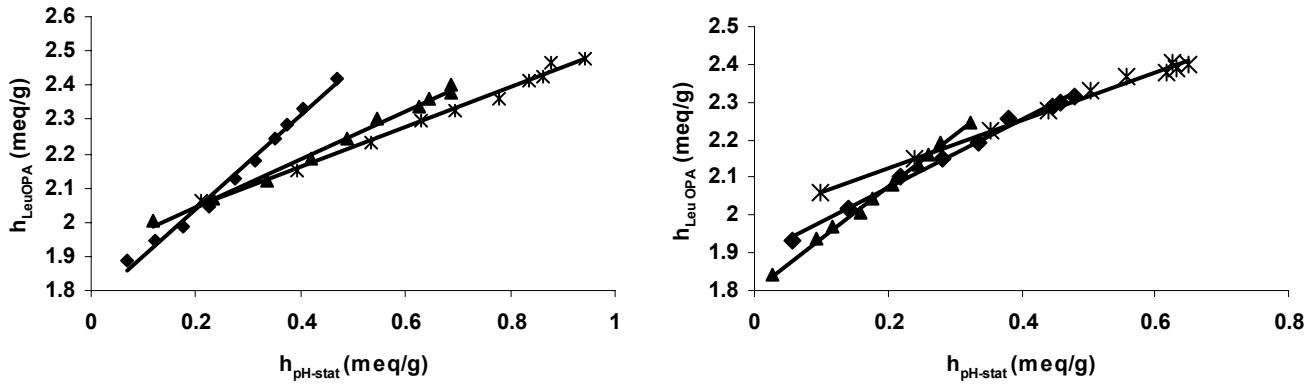



Fig. 1 – Calibration of pH-stat for trypsin (**a**) and chymotrypsin (**b**) hydrolysates of β-LG at 37°C and pH 7 (▲), 7.5 (■) and 8 (★).

At 37°C (the hydrolysis temperature used in all the enzymatic experiments), the average pK for the α-amino groups were 7.18 for trypsin and 7.21 for chymotrypsin. The calculated values of pK for the different combination of pH were significantly different, as it can be seen in Table 1. We assume that pK value of the α-amino groups released is probably affected by the pH-induced critical conformational changes of β-LG. Indeed, β-LG undergoes pH-dependent dissociation/association reactions (well-known Tanford transition). In the 3.5-5.2 pH range, β-LG exists predominantly as an octamer, between 5.2 and 7.0 preferably as a dimer, and at pH > 7.0 the dimer dissociates to the monomer.[18]

The variation in pK values obtained by a combination of three pH may reflect that pK is the average of a range a pK values. For example, if we combine pH 7.0 and 8.0 for chymotrypsin hydrolysis, a higher pK value is obtained (pK 7.38). The maximum pK value for the trypsin hydrolysis was obtained when we combine pH 7.5 and 8.0. This can be explained by a slight-over consumption of base at pH 8.0 due to the partial titration of originally buried now exposed tyrosine residue, for which pK is about 9.6. In contrast, a minimum pK value was obtained when we combine pH 7 and 7.5 (6.99) and 7.5 with 8.0 (7.02) for trypsin and chymotrypsin, respectively. Thus, a mean value was taken into account for the calculation of the degree of hydrolysis (pK=7.19). Based on equation 2, the degree of dissociation (α), and subsequently the proportionality constant at pH 7 and pH 7.5 were calculated. Values for $\alpha^{-1}$ were 2.57 and 1.5 for enzymatic hydrolysis at pH 7 and 7.5, respectively. The parameter $h_{tot}$ was calculated from amino acids analysis by summing the mmoles of each individual amino acid per mg of protein. The $h_{tot}$ for β-LG is $8.75 \cdot 10^{-3}$ meq/mg proteins.

*Table 1*

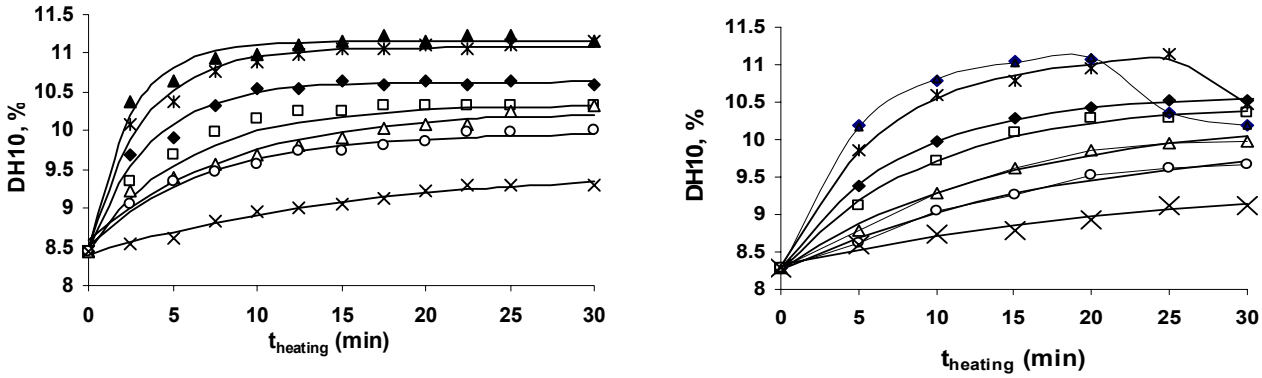
Calculation of pK values for amino groups in β-LG hydrolysates at 37°C

| Enzyme | pH | Equation | Corr coef, $r^2$ | Slope | pK | |
|---|---|---|---|---|---|---|
| | | | | $b_1$ | $b_2$ | |
| **Trypsin** | 7 | y=1.3637x+1.8043 | 0.9964 | 1.3637 | 0.8983 | 6.99 |
| | 7.5 | y=0.8983x+1.8939 | 0.9936 | 0.8983 | 0.6352 | 7.38 |
| | 8 | y=0.6352x+1.9973 | 0.9919 | 1.3636 | 0.6352 | 7.18 |
| | | | | | **mean** | **7.18** |
| **Chymotrypsin** | 7 | y = 1.3538x + 1.9295 | 0.990 | 1.3538 | 0.697 | 7.38 |
| | 7.5 | y = 0.697x + 1.907 | 0.993 | 0.697 | 0.5784 | 7.02 |
| | 8 | y=0.5784x+1.9295 | 0.9912 | 1.3538 | 0.5784 | 7.24 |
| | | | | | **mean** | **7.21** |

## The effect of thermal treatment on the susceptibility of β-LG to enzymatic hydrolysis by trypsin and chymotrypsin

During the pH-stat calibration experiments, we observed a significant difference between the base consumption for the hydrolysis experiments conducted at pH 7 and 7.5 for both enzymes studied. We assumed this to be due to the pH-induced molecular unfolding, resulting in an increased surface area accessible to the proteolytic enzymes. In this context, the actual hydrolysis experiments on heat treated samples were conducted at these two different pH values (7 and 7.5).

To investigate the effects of heat treatments temperature on the subsequent susceptibility of β-LG to proteolysis, samples were heated at various constant temperatures for 0-30 min. It should be noted that all heating experiments were performed at a single pH=7. Under the conditions applied here (3.8 mL of aqueous solutions, 2.5 mg/mL β-LG), no increase in turbidity of the β-LG solutions was observed, not even after 30 minutes of heating at 78°C. β-LG was less resistant to tryptic hydrolysis even in the native state, due to higher substrate accessibility resulting from partial dissociation of the protein molecules. The initial DH (untreated sample) obtained after 10 min of enzymatic reaction at 37°C was 8.43±0.24% and 8.3±0.23% at pH 7 and 7.5, respectively. These values are not surprising, considering the high E:S ratio used in these studies. Heat-treatments of β-LG solution between 62.5-78°C result in structural unfolding of protein conformation that increased the subsequent extent of proteolysis by trypsin. As can be seen in Fig. 2, trypsin gave an extent of proteolysis not very different at pH 7 and 7.5 on heat-induced modified β-LG solutions.




**a)** **b)**

Fig. 2 – Heat induced changes in susceptibility of β-LG to trypsin hydrolysis at pH 7 (a) and 7.5 (b) (×62.5°C, o 65°C, Δ 67.5°C, 70°C, ◆ 72.5°C, ∗ 75°C, ▲ 78°C).

Longer treatment time resulted in a higher extent of susceptibility to proteolysis, until an equilibrium level was reached. The maximum value for DH (11.22%) was reached after 20-25 min

of heating at 78°C, probably because at prolonged heating time, the molecular association is favoured. The above mentioned observation can be explained considering that upon heating at higher temperature and neutral pH, the molecules are involved in polymerization via sulphydryl-disulfide interchange reactions and hydrophobic interactions. Extensive aggregation minimized the accessibility of the unfolded proteins to the hydrolytic enzymes. Iung *et al.,* (1991) found also an increase in degree of hydrolysis with trypsin at 37°C and pH 7.5, for β-LG that was heat-treated at 90°C. The highest DH value (11%) was obtained after 2 h of hydrolysis at a lower E:S ratio (1:25), compared with this study.

The hydrolysis degree of the samples as determined by base consumption during 10 min of hydrolysis at constant pH by chymotrypsin showed a difference between native and heated β-LG (Fig. 3). The initial DH10 obtained by hydrolysis at 37°C was found to be 13±0.24% and 11.34±0.11% at pH 7 and 7.5, respectively, meaning that β-LG was sensitive to enzymatic hydrolysis at both pHs. Upon heat-treatment between 62.5-78°C, the hydrogen bonds and hydrophobic interactions are disrupted, increasing the dissociation of β-LG dimer. The dimer dissociation results in reversible conformational changes at pH 7.5, causing the exposure of the four tryptophan residues and two of the four tyrosine residues, therefore exposing the sensitive peptide bonds for the action of chymotrypsin.

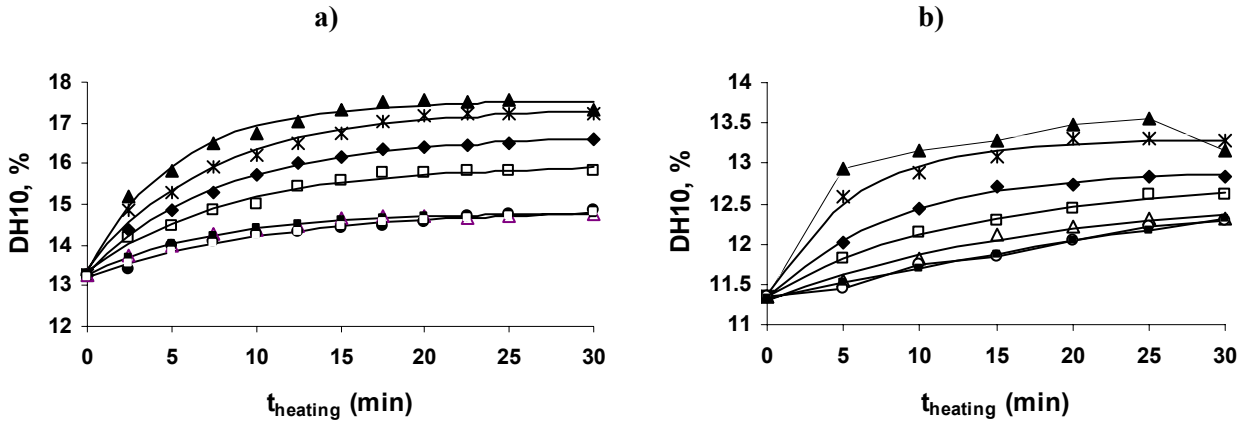



Fig. 3 – Heat induced changes in susceptibility of β-LG to chymotrypsin hydrolysis at pH 7 (a) and 7.5 (b) (×65°C, Δ 67.5°C, 70°C, ◆ 72.5°C, ∗ 75°C, ▲ 78°C).

The DH gradually increases with temperature. In a lower temperature range (62.5-65$^0$C), the increase in DH10 was not significant. This confirms earlier reports on the reversibility of heat-induced structural modifications in subdenaturing conditions.[12,19] The slightly increase in DH10 in these conditions could indicate possibly that some of the residues specifically recognized by each protease were exposed, without irreversible structural modification.

From the hydrolysis curves at pH 7.5 for heat-treated β-LG solutions was shown that the hydrolysis rate is faster in the first 100 sec of reaction for β-LG that was treated for 5-15 min compared with un-treated protein (data not shown). At higher temperature (78$^0$C), a first, strong increase in susceptibility was observed, followed by a decrease after 25-30 minutes of heating, probably due to the formation of less soluble aggregates, inaccessible to hydrolytic enzymes. This means that the substrate is changing as the dimer dissociates to monomer. Upon heating at prolonged time, the dimer dissociation directly coincides with an increase in reactivity of the free thiol groups and hydrophobic sites, which react to form a covalent/noncovalent dimer, trimer, etc.

The highest DH obtained with chymotrypsin, at a higher E: S ratio used in this study, was 17.2% for the protein that was heated at 78°C for 25 min. The temperature-dependent increase in susceptibility of β-LG to chymotrypsin proteolysis is consistent with the results of Reddy *et al.,* (1988), who observed a marked increase in susceptibility of β-LG for chymotrypsin hydrolysis for the protein that was heated at 80°C and 90°C.

Thermal treatment was shown to promote conformational changes of β-LG structure at neutral pH, inducing the dissociation of dimer with

the exposure of previously buried hydrophobic groups. The DH10 for the native protein was significantly higher for the chymotrypsin than that for trypsin, for both pH values studied (*e.g.*, 13% compared with 8.4% for the hydrolysis experiments at pH 7). Given the differences in specificity, the higher DH10 obtained with chymotrypsin indicates that heat-treated β-LG solutions undergo extensive conformational changes as the pH is adjusted at 7.5, resulting in the exposure of the strategic peptide bonds for the enzyme. These cleavages induce further structural changes eventually allowing cleavage at many points. In this context, it seems theoretically normal and actually observed (Fig. 4) that chymotrypsin gave a higher extent of hydrolysis levels compared with trypsin, for all temperatures studied, including untreated β-LG.

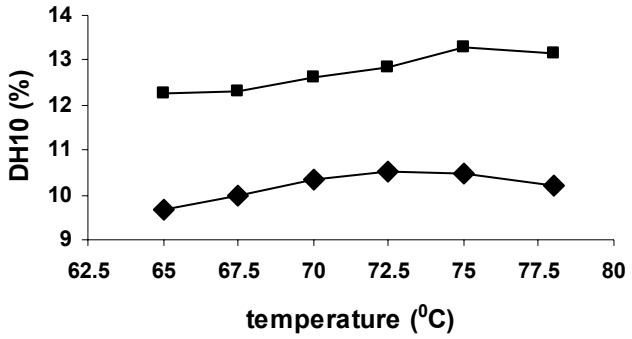



Fig. 4 – Heat-induced changes in enzyme susceptibility of β-LG solutions treated for 30 minutes at different temperature (♦trypsin, ■ chymotrypsin).

## Kinetics of heat-induced changes in enzymes susceptibility of β-LG

The time-dependent changes in susceptibility of β-LG to enzymatic hydrolysis are showed in Figs. 2 and 3, for different thermal treatments. The increase in DH10 due to thermal treatment could be described by a first-order fractional conversion kinetic model. The rate constants at different temperatures of the fractional conversion model (confidence level P = 95%) for thermal denaturation of β-LG as measured by DH10 are given in Table 2.

*Table 2*

Kinetic parameters k and $E_a$ estimated by the first order fractional conversion model describing heat-induced changes on the susceptibility β-LG to enzymatic hydrolysis at different pH

| | **k ($min^{-1}$)** | | | |
|---|---|---|---|---|
| | **Trypsin** | | **Chymotrypsin** | |
| **Temperature (°C)** | **pH 7** | **pH 7.5** | **pH 7** | **pH 7.5** |
| **62.5** | 0.062±0.012[a] | 0.04±0.019 | nd | nd |
| **65** | 0.149±0.017 | 0.058±0.012 | 0.084±0.015 | 0.017±0.014 |
| **67.5** | 0.112±0.021 | 0.072±0.01 | 0.134±0.01 | 0.054±0.018 |
| **70** | 0.175±0.009 | 0.108±0.01 | 0.119±0.012 | 0.077±0.009 |
| **72.5** | 0.270±0.023 | 0.133±0.003 | 0.127±0.006 | 0.121±0.009 |
| **75** | 0.308±0.027 | 0.158±0.014 | 0.138±0.011 | 0.17±0.021 |
| **78** | 0.414±0.039 | 0.218±0.007 | 0.190±0.015 | 0.296±0.081 |
| **$E_a$ ($kJ.mol^{-1}$)** | **122.54±7.52** | **105.8±4.7** | **61.8±3.2** | **161.1±3.9** |

[a] : values ± standard errors of regression.

At lower temperature (62.5-65°C), the influence of temperature on the trypsin susceptibility is lower compared with higher temperature (Fig. 2). This indicates that heating β-LG solutions in this range induced reversible changes in protein conformation, as measured by the changes in susceptibility to enzymes hydrolysis. It can be seen that *k*-values for the increase in trypsin

susceptibility of β-LG at pH 7 are approximately 2 times-higher than those for pH 7.5, especially at higher temperature. In the temperature range of 62.5-78°C, the temperature dependence of the rate constants could be described by the Arrhenius model (equation 5), resulting in an activation energy of 122.6±7.6 kJ/mol ($r^2$ = 0.985) and 105.8±4.7 kJ/mol ($r^2$ = 0.989), at pH 7 and 7.5, respectively (Fig. 5).

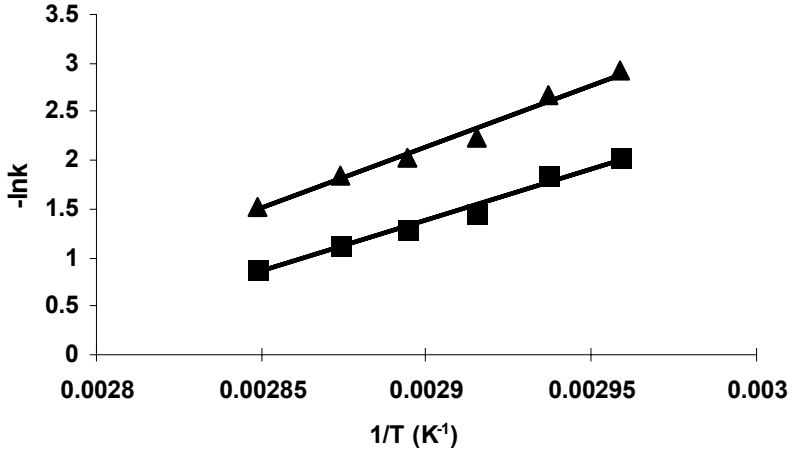



Fig. 5 – Temperature dependence of the rate constants of the fractional conversion model describing the heat-induced changes in susceptibility of β-LG to trypsin hydrolysis at pH 7 (■) and 7.5 (▲).

As for chymotrypsin, the time-dependent effect of temperature on the susceptibility of β-LG to chymotrypsin hydrolysis is depicted in Fig. 3. As can be seen, the thermal treatment had a more pronounced influence on the chymotrypsin susceptibility especially when measured at pH 7.5. Longer treatment times resulted in an increase in DH10, until an equilibrium value was reached. By monitoring the kinetics of heat induced changes in chymotrypsin susceptibility of β-LG, we observed that the *k*-values were significantly higher for the enzymatic hydrolysis at pH 7, compared with hydrolysis at pH 7.5, except at 75 and 78°C (Table 2). In the temperature range of 65-78°C, the temperature dependence of the rate constants could be described by Arrhenius equation, as depicted in Fig. 6. The resulting activation energy was 61.8±3.2 kJ/mol ($r^2$ = 0.992) and 161.1±3.83 kJ/mol ($r^2$ = 0.998) for the susceptibility to chymotryptic hydrolysis at pH 7 and 7.5, respectively.

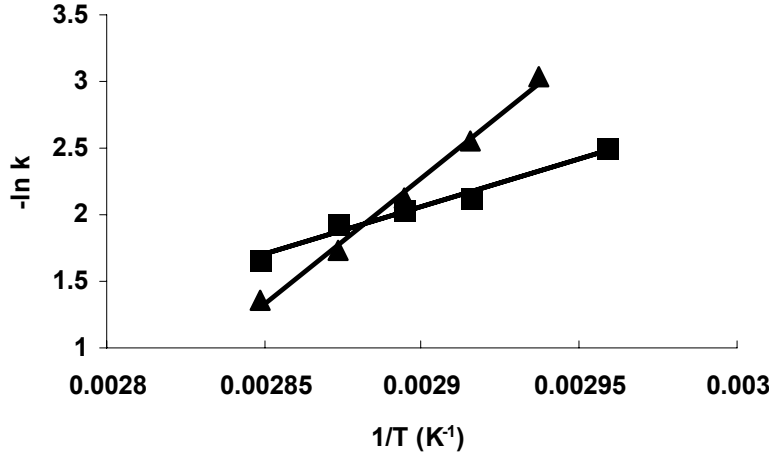



Fig. 6 – Temperature dependence of rate constants of the fractional conversion model describing the heat-induced changes in susceptibility of β-LG to chymotryptic hydrolysis at pH 7 (■) and 7.5 (▲).

The first order fractional conversion model was characterized by an end value of the DH10 after prolonged heating time. At lower temperatures, this equilibrium values was temperature dependent, indicating a temperature-dependent level of denaturation. The existence of such equilibrium

values implies that for each temperature, a treatment time can be establish after which longer heating time does not result in changes in the DH.

The observed differences in $k$ values and $E_a$ are probably determined by the changes in the substrate when the pH is changed from 7.0 cu 7.5 after thermal treatment. Elsewhere, we have been shown that upon heating β-LG at neutral pH, the native dimers start to dissociate into monomer, leading to the exposure of the previously buried hydrophobic amino acids and the single free thiol group. Above 78°C, this is accompanied by the aggregation of the β-LG molecules as a result of sulphydryl-disulfide interchange reactions and hydrophobic interactions, with consequences for protein solubility.[20]

## CONCLUSIONS

Susceptibility to enzymes hydrolysis was used in this study as an index of molecular flexibility. Thermal treatment caused partial unfolding of protein molecules and increased hydrolysis rate of hydrolysis of β-LG by both trypsin and chymotrypsin at 37°C. This phenomenon is due to the fact that accessibility of the specific peptide bonds to the enzymes is enhanced. At 78°C, the decrease in DH10 after prolonged heating time is probably due to the association/aggregation of the molecules, which can hide some susceptible bonds for the enzymes.

The time-dependent changes in the susceptibility of β-LG to enzymes hydrolysis could be described by a first order fractional conversion model. The values for activation energy were two times higher for the susceptibility of β-LG to trypsin hydrolysis compared with chymotrypsin hydrolysis at pH 7. In contrast, for the hydrolysis experiments at pH 7.5, β-LG seems to be more heat-sensitive for chymotrypsin hydrolysis, confirming that the dimer dissociates when the pH is adjusted at 7.5, with the exposure of hydrophobic residues. Also, the decrease in DH10 was observed only for the hydrolysis experiments at pH 7.5, after prolonged heating time at higher temperature, for both enzymes studied. This observation confirms the earlier results that the highly reactive monomers formed during heat-treatment associate to form noncovalent/covalent aggregates, which can hide the specific peptides bonds for the enzymatic cleavage.

These time-dependent changes in susceptibility of β-LG to enzymatic hydrolysis can be an indicator for increased flexibility due to heat treatment. As a result, the surface functional properties can be modified such as surface hydrophobicity, solubility and turbidity. Additionally, the hydrolysis of β-LG with trypsin is known to generate bioactive peptides and also peptides with emulsifying properties. As different final levels of DH10 after thermal treatment were observed for both enzymes studied, differences in solubility and foaming properties are also expected after a limited proteolysis.

These results indicate that the susceptibility of β-LG to proteolysis can be modified by thermal treatment, and it can be important not only for pasteurization or sterilization of milk, but also for modification of the functional properties of whey proteins as a potential ingredient in different food systems.

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