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Original article

Reaction kinetic pathway of reversible and irreversible thermal denaturation of β-lactoglobulin

Alexander TOLKACH*, Ulrich KULOZIK

Institute for Food Process Engineering and Dairy Technology, Technical University Munich, Weihenstephaner Berg 1, 85354 Freising-Weihenstephan, Germany

Abstract – A reaction kinetic approach for the description of reversible (unfolding) and irreversible (aggregation) denaturation of β-lactoglobulin (β-Lg) was developed in this study. This novel approach allows the calculation of the content of native, partially unfolded and irreversibly denatured β-Lg as a function of the heat treatment time. Moreover, it is able to give a mathematical interpretation of the sharp bend on the Arrhenius plot of the formal denaturation rate constant which is characteristic of the denaturation behaviour of both major whey proteins, α-lactalbumin and β-lactoglobulin. The developed theoretical backgrounds were applied for the denaturation kinetics of β-Lg in a protein solution containing 50 g·L^{-1} of the isolated whey protein fraction and verified by means of differential scanning calorimetry.

β-lactoglobulin / thermal denaturation / unfolding / aggregation / reaction kinetic

摘要 – β-乳球蛋白可逆和不可逆热变性反应动力学。本文研究了β-乳球蛋白（β-Lg）可逆（去折叠）和不可逆（凝聚）热变性反应动力学方法。该种新方法可以根据热处理时间计算天然的、部分去折叠的和不可逆热变性的β-乳球蛋白含量。此外, 根据变性速率常数的阿累尼乌斯图中的拐点可以从数学上解释乳清蛋白中 α-乳白蛋白和 β-乳球蛋白的变性行为。此种新建立的理论方法已经用于解释浓度为 50 g·L^{-1} 的乳清分离蛋白溶液中β-乳球蛋白的变性动力学, 并采用差示扫描量热法进行了验证。

β-乳球蛋白 / 热变性 / 去折叠 / 凝聚 / 反应动力学

Résumé – Cinétique de la réaction de dénaturation réversible et irréversible de la β-lactoglobuline (β-Lg). Une approche de la cinétique de réaction pour décrire la dénaturation réversible (dépliement) et irréversible (agrégation) de la β-Lg a été développée dans cette étude. Ce nouveau modèle cinétique permet de calculer la fraction de β-Lg native, partiellement dépliée et irréversiblement dénaturée en fonction de la durée de traitement thermique. De plus, l'approche cinétique développée est capable de donner une interprétation mathématique du brusque changement de pente de la constante de dénaturation en fonction de la température, caractéristique du comportement de dénaturation des deux protéines sériques majeures, α-lactalbumine et β-lactoglobuline. Le modèle théorique développé a été appliqué à l'étude de la cinétique de dénaturation de la β-Lg dans une solution protéique à 50 g·L^{-1} de la fraction de protéine sérique isolée, et vérifié par mesure en calorimétrie différentielle.

β-lactoglobuline / dénaturation thermique / dépliement / agrégation / cinétique de réaction

* Corresponding author (通讯作者): Alexander.Tolkach@wzw.tum.de

1. INTRODUCTION

Bovine β-lactoglobulin (β-Lg) is a major whey protein with a molecular weight of 18.3 kg·mol^{-1} and average concentration in milk or whey of 3.5 g·L^{-1} [37]. Under physiological conditions the protein exists as a dimer. It belongs to the proteins of the lipocalin family that have an ability to bind and transport small molecules within a central hydrophobic core [28]. The amino acid sequence of β-Lg comprises 162 residues that marginally differ depending on the genetic variant of the protein. Currently, 13 genetic variants of β-Lg are known, whereas two variants, β-Lg B and β-Lg A ($Gly_{64} \rightarrow Asp_{64}$, $Ala_{118} \rightarrow Val_{118}$), are the most common ones [28]. The molecular structure of β-Lg consists of 6–10% α-helix, 44–52% β-sheet, 8–10% turn and 32–35% of random coil [4,12,26]. The tertiary structure is strongly dependent on temperature, pH and other environmental conditions and is stabilised by two disulphide bonds (Cys_{106}-Cys_{119} and Cys_{66}-Cys_{160}). β-Lg also has a free thiol group (Cys_{121}), which is normally hidden by the α-helix in the native state (Fig. 1).

Due to its excellent functional properties, this protein has found wide applications in the food industry as a texturing agent and a structure stabiliser [23]. The functional properties of β-Lg are strongly affected by its ability to denature and to react with thermally stable proteins such as caseins [5] as well as to form aggregates with diverse structures [32] or conjugates with other milk, whey or egg proteins [22, 29, 39]. Thermal denaturation of β-Lg appears to be an essential technique to improve the functionality of this protein for its use in the different applications of food manufacture.

The mechanism of thermal denaturation of β-Lg is the subject of a considerable number of interesting studies [2, 13, 15, 20, 25, 26] that resulted in a number of hypothetical models describing the thermal behaviour of β-Lg in heated solutions assessed by different analytical techniques. By summarising the previous studies the thermal denaturation of β-Lg can be characterised as a multistage process, depicted in Figure 2. According to this scheme the native dimer of β-Lg (N_2) dissociates reversibly into native monomers (N) at temperatures above 40 °C. In the temperature range between 40 and 55 °C the monomer undergoes an intramolecular transition into a so-called R-state (N_R), which is especially characteristic for pH values above 7.5. The R-state of β-Lg differs from the native state only by minor conformational changes of some side chains (especially the α-helix that masks the free thiol group) and, consequently, by enhanced accessibility of the free thiol group. The major secondary structure of the molecule remains nearly unaltered and very similar to the native one. The β-Lg molecule in the R-state is able to form polymers. However, the polymerisation rate is negligible.

When the temperature of the protein solution increases above 60 °C, the β-Lg molecule undergoes a reversible partial unfolding and forms a thermally induced molten globule state (U_{MG}). The critical transition of β-Lg contains a complete unfolding of the α-helix structure and exposition of the free thiol group (Cys_{121}) and some parts of the hydrophobic core (see Fig. 1). However, at this point partially unfolded β-Lg molecules with exposed Cys_{119} can also be formed by intramolecular interchange reactions. This form is not able to refold into the native state, which results in the formation of irreversible β-Lg monomers upon cooling [6, 30]. Via the hydrophobic bonds and thiol exchange, β-Lg molecules take part in the irreversible intermolecular interactions that result in the formation of polymers and aggregates (U_n, U_m). Most authors distinguish between polymerisation

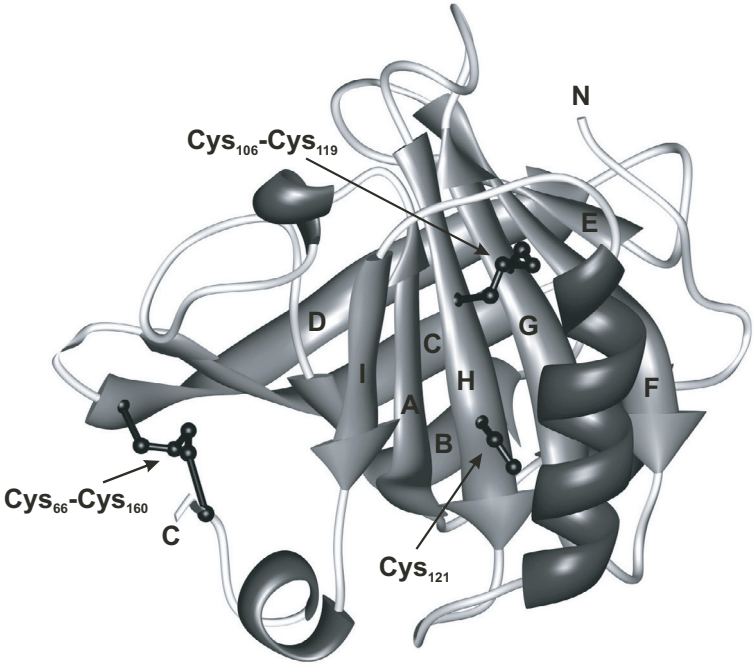

Figure 1. Molecular structure of β-Lg. Molecular graphics images were produced using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIH P41 RR-01081) [24]. Protein data bank file 1B0O was used for the model construction [38].

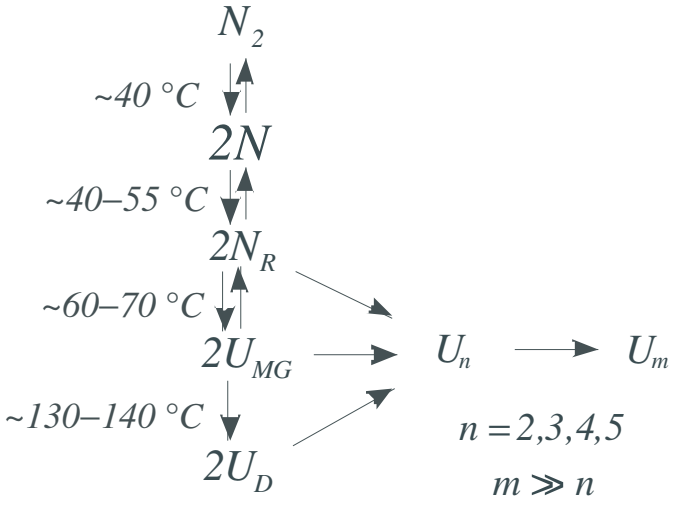

Figure 2. Mechanism of the thermal denaturation of β-Lg.

(formation of polymers by covalent bonds) and aggregation (formation of aggregates by non-covalent interactions) despite the fact that both processes occur simultaneously, leading to the formation of mixed aggregates stabilised by both covalent and non-covalent bonds. The aggregation and polymerisation rate is strongly dependent on the environmental conditions and temperature [32, 33]. According to differential scanning calorimetry (DSC) measurements, the partial unfolding and formation of the molten globule state is completed at temperatures around 90 °C [11]. A temperature increase up to 130–140 °C leads to a complete destruction of the secondary structure of the β-Lg molecule and irreversible denaturation.

The ability to form a thermally induced molten globule state plays a very important role for technological properties of β-Lg. This partially unfolded β-Lg molecule is able to react with other partially unfolded and aggregated or polymerised whey proteins forming particles and gels [32, 33]; the reaction between partially unfolded β-Lg and casein micelles is exploited in yoghurt production [8,9] and must be avoided during hard cheese production due to the inhibition of rennet-induced gel formation. However, this complex formation might be used for the increase in cheese yield in fresh cheese manufacture [37]. Interactions between partially unfolded β-Lg and hot surfaces during treatment of milk and milk products results in the formation of deposit and fouling of heat transfer surfaces [7].

Due to the technological functions of the partially unfolded β-Lg described above, a kinetic modelling of thermally induced molten globule state formation and degradation, allowing the prediction of its concentration as a function of the heat treatment time, is of high significance. However, direct measurements of the content of the partially unfolded β-Lg in situ (e.g. during heat treatment) are difficult or even impossible to perform. The most disturbing factor is that the molecular structure of the thermally induced molten globule state of β-Lg is identical to that of aggregated molecules and most analytical techniques cannot distinguish in situ between both protein states existing at the same time in the solution. Analytical techniques such as DSC are able to monitor the unfolding process during heat treatment; however, they are not in a position to assess the degradation of partially unfolded β-Lg as a result of the aggregation reaction. Online monitoring of the denaturation reaction by particle measurement seems not to be an effective method since the existing techniques can only assess the aggregation, but cannot distinguish between native and partially unfolded states of β-Lg molecules. Particle measurements, as well as techniques such as high-performance liquid chromatography (HPLC), gel permeation chromatography (GPC), ionic exchange chromatography (IEC), polyamidacryl gel electrophoresis (PAGE), and isoelectric focusing (IEF) for the assessment of the contents of native, monomeric and agglomerated or polymerised proteins after heat treatment are also not able to provide any direct information about the concentration of the thermally induced molten globule state due to its refolding after cooling of the protein solution before performing the analysis.

Owing to the above-mentioned problem of the analytical assessment of the thermally induced partially unfolded state of β-Lg molecule, most studies with a focus on the thermal denaturation of β-Lg did apply analytical techniques to distinguish between the native and the aggregated states of the molecule [16, 18, 34–36]. Therefore, the degradation of native or native-like β-Lg was commonly modelled by formal kinetic means supposing the denaturation to be a two-stage process (native → denatured). According to this assumption the degradation rate of native protein

$-\frac{dC_{HPLC}}{dt}$ as a result of thermal denaturation is directly proportional to the concentration of the protein C_{HPLC} in power of the reaction order n with the denaturation rate constant (or denaturation velocity constant) k_f as a proportional coefficient:

$$-\frac{dC_{HPLC}}{dt} = k_f (C_{HPLC})^n . \qquad (1)$$

The formal reaction order n may not be seen as an order of a classical chemical reaction: the formal reaction order expresses in this case the complexity of the denaturation process and usually takes values for the denaturation reaction of β-Lg between 1 and 2, commonly 1.5. After integration of Equation (1), the following expressions can be used for the prediction of the amount of native β-Lg after heat treatment (for $n \neq 1$):

$$C_{HPLC,t} = \left(C_{HPLC,0}^{(1-n)} + (n-1) \cdot k_f \cdot t\right)^{1/(1-n)} \qquad (2)$$

$$\left(\frac{C_{HPLC,t}}{C_{HPLC,0}}\right)^{(1-n)} = 1 + (n-1) \cdot C_{HPLC,0}^{(n-1)} \cdot k_f \cdot t. \qquad (3)$$

Equation (3) is commonly used for graphical interpretations since the formal denaturation rate constant can be easily obtained from the slope of the line by a simple linear regression. The denaturation rate constant k_f is a function of the composition of the solution and heat treatment temperature. The relationship between the formal velocity constant and heating temperature can be mathematically expressed by the Arrhenius equation:

$$\ln k_f = \ln k_{f0} - \frac{E_a}{R \cdot T} \cdot \qquad (4)$$

Contrary to a classical chemical reaction, which, according to the Arrhenius relationship, can be described by means of a straight line when $\ln k_f$ is plotted versus $\frac{1}{T}$, the velocity constant of the β-Lg denaturation shows a discontinuity in the plot, resulting in a sharp bend or edge [34]. The bend temperature (or transition temperature) divides the Arrhenius plot into two parts: unfolding-limited (low temperature range) and aggregation-limited (high temperature range) reaction. The interpretation of such behaviour of the reaction rate constant is that at temperatures lower than the bend temperature the partial unfolding of β-Lg molecules is slower than their aggregation, so the whole denaturation reaction is limited by the unfolding subreactions. In contrast to that, at temperatures higher than the bend temperature, the aggregation of the thermally induced molten globule state is slower than its formation and, consequently, the resulting denaturation reaction of β-Lg is limited by the aggregation process. The position of the sharp bend strongly depends on the pH, lactose content and other compositional parameters.

The major advantage of the formal kinetic description is that the formal reaction rate constant can be simply calculated from experimental data and used for the prediction of the degree of irreversible denaturation for every time-temperature combination. However, the theoretical background of the formal kinetic description does not take into consideration the existence of the molten globule state and, consequently, is not able to separately characterise the unfolding process, polymer and aggregation formation as well as giving a mathematical interpretation of the sharp bend occurrence. For this reason, a number of other models for quantitative description of the subreaction of β-Lg denaturation were adapted or developed. At temperatures between 60 and 70 °C and moderate protein concentration the denaturation of β-Lg could be described in terms of classical polymerisation with initiation, propagation and termination steps [27], fractal theory [1], Fuchs-Smoluchovski kinetics [13] and different numerical models of colloidal aggregation [14, 15]. These mathematical models deliver a very good numerical interpolation of data obtained

by dynamic light scattering or photon correlation spectroscopy in the conditions mentioned above. Nevertheless, they are mainly unable to give a quantitative description of thermally induced β-Lg denaturation over a broad range of temperatures as well as mathematical interpretation of the sharp bend on the Arrhenius plot. Moreover, none of the models was able to calculate the concentration of each of the three β-Lg main states involved in the denaturation process: native, partially unfolded and irreversibly denatured.

Based on this situation, the major objective of this study was to develop a novel reaction kinetic approach that is able to separately describe the reversible unfolding and irreversible denaturation of β-Lg in heat-treated solutions. Therefore, using this reaction kinetic approach, a derivation of mathematical expressions describing the concentration of native, partially unfolded and irreversible aggregated or polymerised β-Lg as a function of heat treatment time was aimed at.

The model development was based on the formal kinetic data generated by ex situ measurements. In this study we decided to use HPLC for the assessment of β-Lg content since this technique provides information about protein molecules remaining native or refolded to the native state after heat treatment. Application of formal kinetic data for development of a novel reaction kinetic approach allows the consideration and interpretation of the phenomena such as the sharp edge on the Arrhenius plot observed by prior kinetic studies.

2. MATERIALS AND METHODS

β-Lg AB was obtained from pasteurised skim milk using the procedure described by Maubois et al. [21]. After freeze-drying, the β-Lg powder was dissolved in ultrafiltration permeate (molecular weight cut-off of the UF membrane 10 $kg \cdot mol^{-1}$, pH 6.8, lactose content 45 $g \cdot L^{-1}$) to obtain a solution containing 50 $g \cdot L^{-1}$β-Lg and left overnight at 4 °C. The concentration of 50 $g \cdot L^{-1}$ was chosen due to the intended DSC measurements in order to obtain a maximal signal/noise ratio.

For the thermal treatment and the evaluation of kinetic data two different systems were used. For temperatures below 100 °C a heat treatment in stainless steel tubes (inner diameter of 3 mm and length of 258 mm) in a water bath was used. The geometry of the tubes allowed heating up the sample within 10 s by application of another water bath for pre-heating (as a rule the temperature of the pre-heating bath was 10 °C higher than the target temperature). Following heat treatment the samples were cooled down immediately in a water bath with melting ice.

For heating temperatures above 100 °C and holding times longer than 60 s a steam-heated pressure vessel was used. The thermal treatment was carried out in the same stainless steel tubes as used for the water bath experiments. In order to minimise the heating-up time the steam pressure during the heating-up was adjusted such that a temperature of 10 °C above the intended heat treatment temperature was realised.

The β-Lg content was determined by HPLC as described in detail by Beyer and Kessler [3]. According to this method, only native β-Lg which showed an identical retention time to the standard fractions obtained from Sigma (β-Lg A: L7889, β-Lg B: L8005) could be detected. Any changes in the tertiary structure of the protein resulted in a shift of the retention time and, consequently, these monomeric proteins with altered structures could not be detected as native by this method. The denatured β-Lg was separated from the solution by shifting the pH value to 4.6 followed by a centrifugation step (10 000× g, 10 min). The supernatant containing native β-Lg was filtered through a membrane filter (pore ø 0.45 μm, Chromafil®

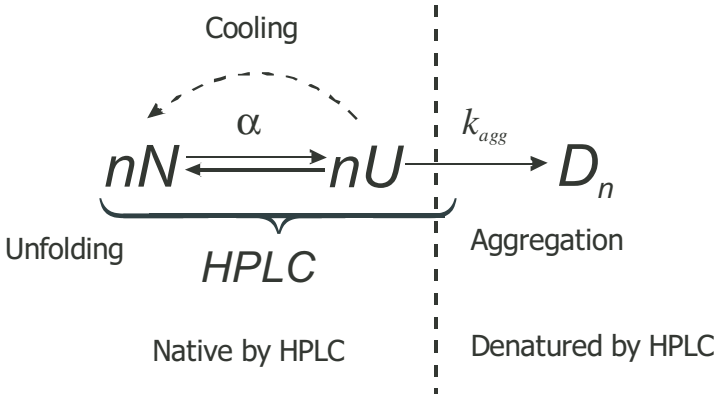

Figure 3. Denaturation of β-Lg as a three-state process.

RC-45/25, Macherey-Nagel, Düren, Germany).

The DSC measurement was carried out using the DSC Q1000 (TA Instruments, Alzenau, Germany). The pans used for the DSC measurements were high-volume pans made of stainless steel with a sample volume of 75 mg. The reference sample was UF permeate. The heating rate used was 5 $K \cdot min^{-1}$.

3. RESULTS AND DISCUSSION

3.1. Development of the mathematical model

According to the scheme in Figure 2 the heat-induced denaturation of β-Lg at temperatures above 60 °C can be simply characterised by a three-state model that involves native molecules of β-Lg, a thermally induced molten globule state (reversible unfolded molecules) and irreversibly denatured (aggregated or polymerised) molecules. Since the aggregation of the R-state is negligible, all reversible reactions in Figure 2 can be substituted by one irreversible unfolding step. Thus, for the kinetic approach this process can be simply described by the reaction equation depicted in Figure 3 which shows the heat denaturation as a two-step process: after reversible unfolding of the native β-Lg molecule (***N***) follows an irreversible aggregation or polymerisation of the thermally induced molten globule state (***U***) with formation of covalent and non-covalent bound protein polymers or aggregates ($\boldsymbol{D}_n$). The stoichiometric coefficient n corresponds to the formal reaction order n in Equation (1) and expresses the complexity of the polymerisation and aggregation processes.

If the temperature of the protein solution drops under 60 °C, the protein molecules in the partially unfolded state ***U*** refold to the native state ***N***, whereas the molecules in the irreversible denatured state $\boldsymbol{D}_n$ are unable to refold. Therefore, analytical techniques such as HPLC, which detect only the amount of the native proteins in the solution after heat treatment, assess all protein molecules that were in the native and reversibly unfolded states at the moment when the temperature dropped below 60 °C:

$$C_{HPLC} = C_N + C_U. \tag{5}$$

The formation of the molten globule state during thermal treatment of protein solution can be characterised by equilibrium between native and partially unfolded β-Lg that quantitatively can be described by an

unfolding degree α:

$$\alpha = \frac{C_U}{C_N + C_U} = \frac{C_U}{C_{HPLC}} \tag{6}$$

or

$$C_U = \alpha \cdot C_{HPLC} \tag{7}$$

and, consequently,

$$C_N = (1 - \alpha) \cdot C_{HPLC}. \tag{8}$$

The irreversible aggregation and polymerisation of the molten globule state of β-Lg can be described by an aggregation velocity constant k_{agg}. Thus, the degradation rate of partially unfolded β-Lg as a result of the aggregation process can be expressed as follows:

$$-\frac{\mathrm{d}C_{U,agg}}{\mathrm{d}t} = k_{agg}\,(C_U)^n\,. \tag{9}$$

From Figure 3 it can be seen that the decrease in β-Lg content assessed as native by HPLC is equal to that caused by aggregation or polymerisation of the molten globule state:

$$\frac{\mathrm{d}C_{HPLC}}{\mathrm{d}t} = \frac{\mathrm{d}C_{U,agg}}{\mathrm{d}t}. \tag{10}$$

Therefore, Equation (9) can be rewritten as follows:

$$-\frac{\mathrm{d}C_{HPLC}}{\mathrm{d}t} = k_{agg}\,(C_U)^n\,. \tag{11}$$

By taking into consideration the relation between the concentration of partially unfolded β-Lg and β-Lg being assessed by HPLC as native (Eq. (7)), differential Equation (11) can be rewritten using the HPLC-assessable content of β-Lg only:

$$\begin{aligned} -\frac{\mathrm{d}C_{HPLC}}{\mathrm{d}t} &= k_{agg}\,(\alpha \cdot C_{HPLC})^n \\ &= k_{agg}\;\alpha^n\,(C_{HPLC})^n\,. \end{aligned} \tag{12}$$

By integration of Equation (12) the content of β-Lg assessed as native by HPLC can be calculated as a function of the heat treatment time:

$$C_{HPLC,t} = \left(C_{HPLC,0}^{(1-n)} + (n-1)\cdot\alpha^n\cdot k_{agg}\cdot t\right)^{1/(1-\mathrm{n})}. \tag{13}$$

By taking into consideration Equations (7) and (8) a calculation of the concentration of unfolded C_U and native C_Nβ-Lg during heat treatment can be obtained from Equation (13):

$$C_{U,t} = \alpha\left(C_{HPLC,0}^{(1-n)} + (n-1)\cdot\alpha^n\cdot k_{agg}\cdot t\right)^{1/(1-\mathrm{n})} \tag{14}$$

$$C_{N,t} = (1-\alpha)\left(C_{HPLC,0}^{(1-n)} + (n-1)\cdot\alpha^n\cdot k_{agg}\cdot t\right)^{1/(1-\mathrm{n})}. \tag{15}$$

Equations (14) and (15) show the relationships between the heat treatment time of the protein solution and the concentration of native ***N*** and partially unfolded form ***U*** of β-Lg. The content of the irreversibly aggregated or polymerised β-Lg can be calculated as the difference between the β-Lg concentration assessable by HPLC and the starting β-Lg concentration. It can clearly be seen that the requirement for the use of the above-mentioned equations is the knowledge of the degree of unfolding α and aggregation rate constant k_{agg}.

The calculation of both α and k_{agg} can be carried out by drawing an analogy between the formal kinetic equation in differential form (Eq. (1)) and the reaction kinetic expression (Eq. (12)), which show a relationship between the rate of decrease of β-Lg that can be assessed by HPLC as native and its concentration. From the comparison of both equations it follows:

$$k_f = \alpha^n \cdot k_{agg}. \tag{16}$$

Or, in logarithmic form:

$$\ln\left(k_f\right) = n\ln\alpha + \ln\left(k_{agg}\right). \tag{17}$$

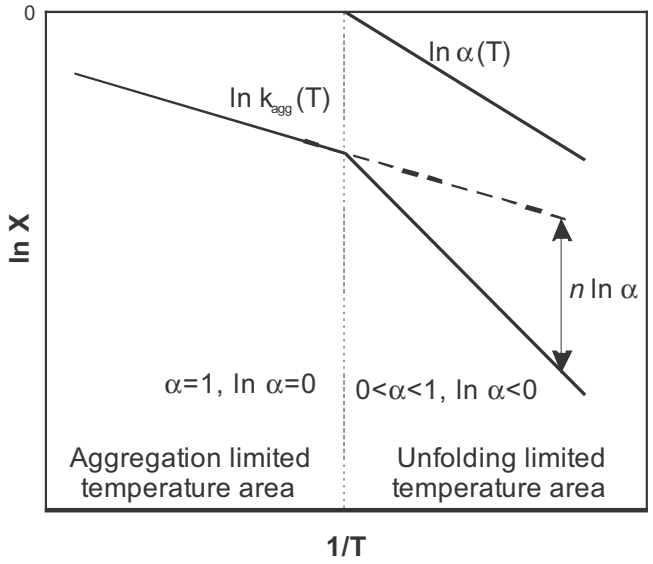

Figure 4. Interpretation of the sharp bend on the Arrhenius plot according to Equation (17).

Equations (16) and (17) show that the formal kinetic rate constant of thermal denaturation of β-Lg can be seen as a direct combination of unfolding and aggregation terms. This allows a mathematical explanation of the sharp edge on the Arrhenius plot of the formal velocity constant (Fig. 4): the value of the unfolding degree α varies between 1 (all protein molecules are completely unfolded, high temperature area) and 0 (all protein molecules are native, low temperature area). Consequently, the value of the term $\ln \alpha$ varies between 0 and $-\infty$. In the aggregation-limited area the unfolding of β-Lg is completed so that only the unfolded and the irreversibly denatured β-Lg molecules exist in the solution. The unfolding degree α takes the value of 1 and, consequently, the term $n \ln \alpha = 0$. Therefore, the formal velocity constant of β-Lg denaturation takes a value of the aggregation velocity constant:

$$\ln\left(k_f\right) = \ln\left(k_{agg}\right). \qquad (18)$$

With decreasing temperature the unfolding degree α declines and the term $n \ln \alpha$ in Equation (17) takes a negative value. Consequently, the relationship between the formal velocity constant and reciprocal absolute temperature deviates from the linear, which results in a sharp edge on the Arrhenius plot. Therefore, in the unfolding-limited temperature area the degree of unfolding α can be calculated from the difference between the formal velocity constant k_f and aggregation rate constant k_{agg} extrapolated into the unfolded limited area:

$$\alpha = \exp\left(\frac{\ln\left(k_f\right) - \ln\left(k_{agg}\right)}{n}\right). \qquad (19)$$

Knowledge of the unfolding degree α and the aggregation rate constant k_{agg} allows the calculation of the content of native, reversibly unfolded and irreversibly denatured β-Lg according to the mathematical expressions mentioned above (Eqs. (14)–(15)).

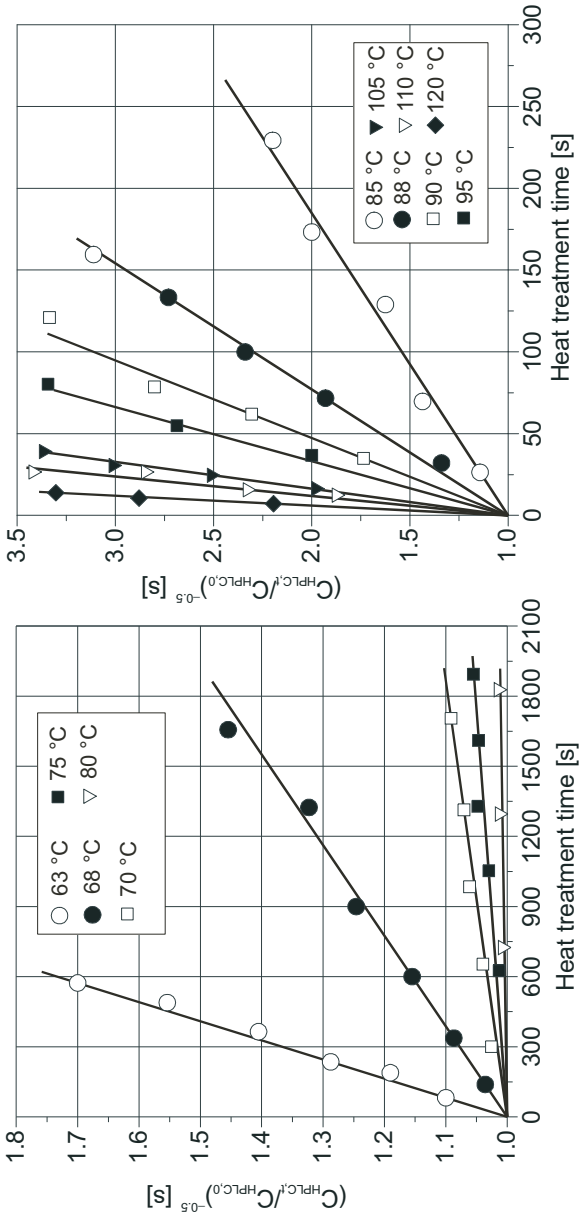

Figure 5. Impact of the heat treatment time and temperature on the loss of β-Lg assessed as native by HPLC.

3.2. Experimental results

Figure 5 shows the impact of the heat treatment time and temperature on the degradation of native β-Lg content assessed by HPLC after cooling down the samples. The relative decrease in β-Lg content is depicted according to Equation (3), whereby the formal denaturation rate constant can be calculated from the slope of the straight line. The formal order n of the denaturation reaction was found to be 1.5 since the results could be linearised by plotting $\left(\frac{C_{HPLC,t}}{C_{HPLC,0}}\right)^{-0.5}$ versus the heat treatment time t. This formal reaction order of the β-Lg denaturation reaction can be well explained by means of the rapid aggregation theory of colloidal solutions [31] or according to the Bodenstein principle of polymer formation reaction [27].

It is evident that with increased temperature the slope of the line and, consequently, the denaturation rate constant increases. The influence of the heat treatment temperature on the formal denaturation velocity constant according to the Arrhenius equation (Eq. (4)) is shown in Figure 6. It can be clearly seen that the relationship between the reciprocal absolute temperature T and the natural logarithm of the formal velocity constant k_f shows a discontinuity in the form of a sharp bend with a transition temperature of 90 °C, dividing the Arrhenius plot into the unfolding-limited and aggregation-limited areas. According to the interpretation given in Figure 4 the unfolding reaction of β-Lg and the formation of the molten globule state must be finished at a temperature below 90 °C. This hypothesis could be proven by a DSC measurement depicted in Figure 6 that shows an endothermic heat flow generated by the unfolding of the β-Lg molecules. It can be seen that the heat flow stops at a temperature of 87 °C, indicating that the unfolding is completed.

Table I shows a calculation of the unfolding degree α using the formal kinetic denaturation rate constant according to Equation (19). As can be seen, the β-Lg molecules start to unfold significantly at a temperature at or above 65 °C, which is in agreement with a number of studies on thermal denaturation of β-Lg [10, 11, 17, 19]. At temperatures below 60 °C the unfolding degree is insufficient for the irreversible aggregation reaction to be started: the probability of a collision between two unfolded β-Lg molecules is extremely low under these conditions.

Knowing the degree of unfolding α and the aggregation rate constant k_{agg} it is possible to predict the content of the native and unfolded β-Lg according to Equations (14) and (15). Figure 7 depicts the influence of the heat treatment time on the concentration of β-Lg assessed as native by HPLC after cooling (measured values) as well as the concentration of the unfolded and native β-Lg (calculated values) at two heating temperatures: 80 °C (unfolding-limited) and 110 °C (aggregation-limited).

According to the reaction equation depicted in Figure 3 the unfolding of β-Lg occurs rapidly until the equilibrium between the native and the molten globule states of the β-Lg molecules is established. The ratio between the concentration of the partially unfolded and native β-Lg under these conditions is given by the unfolding degree α. The decreasing amount of the molten globule state of β-Lg due to the aggregation or polymerisation reaction is balanced by the partial unfolding of the native molecules, with the result that the achieved equilibrium between both states remains unchanged as long as the heating temperature stays constant (80 °C, Fig. 7). Heat treatment of the β-Lg solution at a temperature in the aggregation-limited area (110 °C, Fig. 7) leads to a partial unfolding of all available β-Lg molecules so that only irreversible aggregation takes place. The amount of the partially unfolded protein in

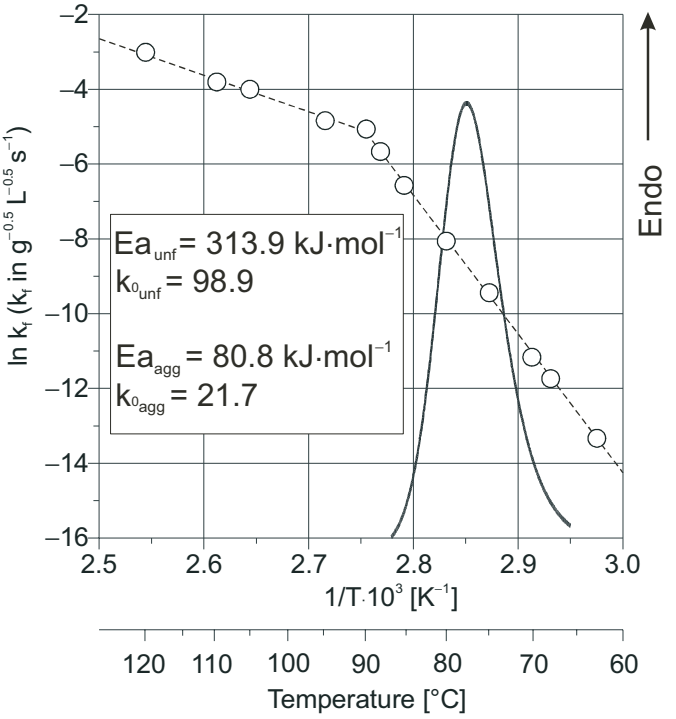

Figure 6. Impact of the heating temperature on the formal denaturation rate constant combined with the DSC thermogram of the β-Lg solution.

Table I. Calculation of the unfolding degree of β-Lg on the basis of the formal kinetic parameters.

Temperature	k_f	$\ln(k_f)$	$\ln(k_{agg})$	$\ln(k_f) - \ln(k_{agg})$	$\ln \alpha$	α
120	4.5×10^{-2}	–3.09	–3.09	0	0	1
110	2.4×10^{-2}	–3.732	–3.732	0	0	1
105	1.7×10^{-2}	–4.066	–4.066	0	0	1
95	8.6×10^{-3}	–4.761	–4.761	0	0	1
90	5.9×10^{-3}	–5.122	–5.122	0	0	1
88	3.7×10^{-3}	–5.602	–5.27	–0.332	–0.22	0.80
85	1.5×10^{-3}	–6.479	–5.50	–0.983	–0.65	0.52
80	3.4×10^{-4}	–7.972	–5.88	–2.092	–1.39	0.25
75	7.4×10^{-5}	–9.509	–6.28	–3.233	–2.15	0.12
70	1.5×10^{-5}	–11.090	–6.68	–4.407	–2.94	0.05
68	8.0×10^{-6}	–11.735	–6.85	–4.886	–3.26	0.04
63	1.5×10^{-6}	–13.382	–7.27	–6.109	–4.076	0.02
60*	5.6×10^{-7}	–14.394	–7.53	–6.861	–4.576	0.01

* Interpolated value.

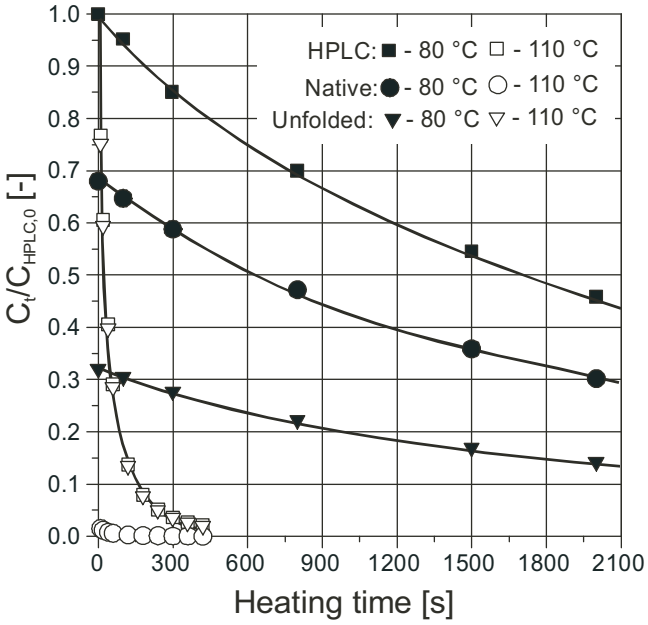

Figure 7. Influence of the heat treatment time on the loss of unfolded, native and HPLC-assessable β-Lg.

this case is equal to the amount of β-Lg assessable by HPLC.

4. CONCLUSIONS AND OUTLOOK

It was shown that the novel reaction kinetic approach for quantitative description of reversible and irreversible denaturation of β-Lg developed above allows the calculation of the content of the native, partially unfolded and irreversibly denatured β-Lg as a function of heating time. The presented kinetic model can be used for independent characterisation of the unfolding and aggregation reactions of β-Lg, as well as other proteins showing a sharp edge on the Arrhenius plot under different environmental conditions. The ability of the denaturation model to quantitatively describe the formation of the molten globule state of globular proteins can be used to model, and for a better interpretation of DSC measurements or the results of other analytical techniques. However, deeper insights into the thermodynamics of the unfolding and aggregation reactions are necessary for that purpose.

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