J Fluoresc (2006) 16:611–621
DOI 10.1007/s10895-006-0088-6

ORIGINAL PAPER

Towards Control of Aggregational Behaviour of α-Lactalbumin at Acidic pH

Jane B. Pedersen · Peter Fojan · John Sorensen · Steffen B. Petersen

Received: 1 December 2005 / Accepted: 23 February 2006 / Published online: 22 June 2006

Abstract α-Lactalbumin (α-La) undergoes considerable structural changes upon loss of bound Ca^{2+} at acidic pH, leaving α-La in a molten globule structure. Using fluorescence the present work provides more insight into the structural transition of α-La at acidic pH leading to protein aggregation, most likely caused by a combination of hydrophobic and electrostatic interactions. The rate of aggregation is determined by the protein concentration and temperature applied. Availability of Ca^{2+} stabilises the protein, and thus prevent aggregation at pH values as low as pH 2.9. In contrast, presence of Cu^{2+} induces a destabilisation of the protein, which can be explained by a binding to the Zn^{2+} binding site in α-La, possibly resulting in structural alterations of the protein. In general, presence of anions destabilise α-La at pH values below pI, with $SO_4{}^{2-}$ exhibiting the strongest effect on the protein stability, thus correlating well with the Hofmeister series. At more acidic pH values far from pI, α-La becomes more stable towards ion induced aggregation, since higher ion activity is required to efficiently screen the charges on the protein surface. The results presented in this paper provide detailed knowledge on the external parameters leading to aggregation of α-La at acidic pH, thus permitting rational design of the aggregation process.

J. B. Pedersen · P. Fojan · S. B. Petersen
Institute of Physics and Nanotechnology, Aalborg University, Aalborg, Denmark

J. Sorensen
Arla Foods amba,
Videbaek, Denmark

S. B. Petersen (✉)
Skjernvej 4C,
9220 Aalborg, Denmark
e-mail: sp@nanobio.aau.dk

Keywords α-Lactalbumin · Protein aggregation · Acrylamide quenching · Fluorescence spectroscopy · Confocal microscopy

Introduction

Cheese whey contains various globular proteins, which are unique in their nutritional and functional properties. To accommodate for the increasing demand for food with improved nutritional and functional properties, much effort has been allocated on examining applicability of whey proteins in food products. The spectrum of food products, utilising whey proteins, is very broad, which places specific demands on the textual properties of the whey proteins, in terms of foam stability, emulsification, water holding capacity and gel formation [1]. This implies requirements for efficient and tight control of the aggregational behaviour of the whey proteins. One major application is the acidic protein beverages, used as a nutritional supplement of essential and branched chain amino acids. Protein beverages require a clear suspension, and the primary prerequisite to success is therefore preventing aggregation of the whey proteins in this application. The aggregational behaviour of whey proteins has been thoroughly studied at basic pH values [2–6]. However, aggregation at acidic pH values is still poorly understood at the molecular level.

In general, protein aggregation is influenced by a number of physical parameters, such as pH, temperature, ionic strength, ion type, pressure, protein concentration and protein composition. Purified whey protein is a highly complex system, where several of the physical parameters might influence the aggregational properties of the proteins in the beverages. This greatly complicates identification of the specific protein responsible for aggregation initiation, which may contribute to gelation. Hence, detailed studies of

individual whey proteins are essential to clarify the events occurring during aggregation of whey proteins at acidic pH.

α-Lactalbumin (α-La) is a small (M_w 14.2 kDa), acidic (pI 4–5), Ca^{2+} binding protein, which constitutes approximately 25% of the proteins in whey [7]. The physiological role of α-La is to support the biosynthesis of lactose in milk through regulation of lactose synthase, which catalyzes the final step in lactose biosynthesis [8]. α-La is widely used as a simple model for Ca^{2+} binding. As pH is shifted towards acidic values α-La becomes progressively more positively charged. At some pH values also the Glu and Asp rich binding pocket for Ca^{2+} is titrated and consequently Ca^{2+} is ejected from the protein. The Ca^{2+} depleted form of α-La attains a classical molten globule state with significantly altered properties [9, 10]. Hereby, T_m is shifted from 62°C at pH 8 to 32°C at pH 2.5 [11]. The formation of the molten globule is likely a prerequisite for protein aggregation, since the hydrophobic interior of the protein becomes more accessible, which is likely to lower the energy barrier for protein aggregate formation. Furthermore, some forms of α-La have recently been found to induce apoptosis in tumor cells suggesting additional significance of this protein [12, 13].

This paper will address the aggregational behaviour of α-La at acidic pH. Hereby, the effect of pH, ions, temperature, protein concentration and presence of Ca^{2+} on conformational stability of α-La is examined through tryptophan fluorescence spectroscopy. Moreover, fluorescence quenching by acryl amide is used for assessing the solvent exposure of tryptophan independent of locally charged residues. Confocal Laser Scanning Microscopy (CLSM) of the α-La in the aggregational state is performed to provide further insight into the effect of ions on the aggregate structure of α-La at acidic pH.

Experimental

Ca^{2+} depleted bovine α-La (L-6010), Acrylamide, Thioflavin T (ThT) and Rhodamine B (RhodB) were obtained by Sigma-Aldrich.

All protein concentrations were determined spectrophotometrically using an extinction coefficient (ε_{280}) of 28,500 M^{-1} cm^{-1}, and adjusted to appropriate pH by HCl.

Fluorescence measurements

Steady-state fluorescence spectroscopy was performed using RTC 2000PTI spectrofluorometer with 90° geometry equipped with a magnetically stirred, thermostated cuvette compartment. All experiments were conducted using 2 nm slit size and tryptophan (Trp) excitation of 295 nm. A measurement of both Trp emission spectra and static light scattering was performed in all experiments to further evaluate the observed results.

Thermal stability

Unfolding was performed by a linear temperature increase of 1.5°C per minute from 5 to 85°C monitoring Trp emission at 350 nm and using a concentration of α-La of 0.6 mM at pH 2.9 and 3.3. Effect of Ca^{2+} binding was observed using a concentration of $CaCl_2$ of 2.5 mM, corresponding to a Ca^{2+} to protein ratio of $\sim$4, thereby assuring excess of Ca^{2+}. Effect of ions on protein stability was observed by applying concentrations of 50 mM of $CuCl_2$ or $CuSO_4$. T_m values were calculated by fitting data to an equation for two-state unfolding [14], see Eq. (1).

$$I = \frac{\alpha_N + \beta_N T + (\alpha_U + \beta_U)\, T\, e^{-\left(\frac{\Delta HT_m}{RT} - \frac{\Delta HT_m}{RT_m}\right) + \frac{\Delta C_p}{RT}\cdot\left(T_m - T + T\ln\left(\frac{T}{T_m}\right)\right)}}{1 + e^{-\left(\frac{\Delta HT_m}{RT} - \frac{\Delta HT_m}{RT_m}\right) + \frac{\Delta C_p}{RT}\cdot\left(T_m - T + T\ln\left(\frac{T}{T_m}\right)\right)}}, \quad (1)$$

where α and β are constants, T is the actual temperature and T_m is the transition midpoint.

Acrylamide quenching

Quenching of Trp was investigated by acrylamide as quencher, 0–0.8 M. Quenching data were analysed by Stern/Volmer plots [15]. The dynamic quencher constants were resolved by the slope of the plot, see Eq. (2).

$$\frac{F_0}{F} = 1 + K_D \times [Q] \quad (2)$$

When examining ion effects 0.25 mM of α-La was utilised at pH 2.0, 2.9 or 3.5, with either 0 or 2.5 mM $CaCl_2$ and 50 mM of either $CuCl_2$, $CuSO_4$, $Cu_3(PO_4)_2$, NaCl, Na_2SO_4 or Na_3PO_4. All samples were prepared at room temperature. Effect of protein concentration and time was investigated at 0.25, 0.5, 0.75 and 1 mM α-La at pH 3.0 without Ca^{2+} present and within a time frame of 0–336 hr, incubation at 30°C.

Confocal laser scanning microscopy

Confocal Laser Scanning Microscopy was performed using a Zeiss LSM510 CLSM equipped with a meta-filter and a 63 × oil objective. ThT and RhodB bound to proteins were excited with an Argon laser (458 nm), a HeNe laser (543 nm) and emission was detected in the interval 473.3–494.7 nm

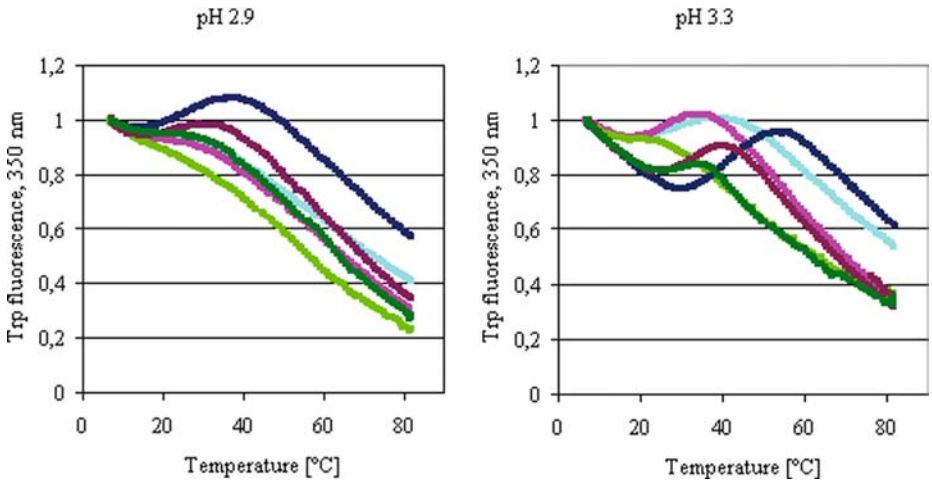

Fig. 1 Thermal unfolding of α-La at pH 2.9 og 3.3 performed in an interval of 5–85°C and 1.5°C/min. With Ca^{2+} present: no ions, $CuCl_2$ and $CuSO_4$. Without Ca^{2+} present: no ions, $CuCl_2$ and $CuSO_4$

and 644–687 nm, respectively. Protein samples of 0.3 mM α-La at pH 3.0 without Ca^{2+} present were incubated at 30°C for 7 days prior to the experiment. The effect of ions on aggregate structure was examined using 50 mM of either $CuCl_2$, $CuSO_4$ or $Cu_3(PO_4)_2$ and α-La was stained with 50 μM of either ThT or RhodB.

Computational titration of α-La

Titration of amino acid residues of α-La in the acidic pH range was performed computationally using Titra software version 1.5, as previously described [17].

Results

Thermal stability

Thermal stability data of proteins provides direct information on the structural effects by different external parameters. Thermal unfolding of α-La has therefore been performed to investigate the stabilisation or destabilisation of pH, ions and presence of Ca^{2+}, see Fig. 1. Hereby, it is expected that both addition of ions and lowering of pH destabilise α-La, whereas presence of Ca^{2+} is expected to stabilise the protein.

Corresponding computed T_m values and enthalpies are presented in Table 1.

The fluorescent unfolding and corresponding enthalpies and T_m values reveal an overall stabilising effect of Ca^{2+} on α-La, whereas both lowering pH and addition of ions destabilise the protein, which is in correlation with expectations. Hereby, destabilisation is reflected in both a decrease in T_m value and less transitional change in fluorescence. As observed, the presence of Ca^{2+} is most effective at pH 3.3 as would be expected. By comparing effects of $CuCl_2$ and $CuSO_4$, $CuSO_4$ exhibit the most destabilising effect on the three-dimensional structure of α-La, as observed from the transition of unfolding occurring to less extent and at a lower temperature.

Acrylamide quenching

Acrylamide is a neutral quencher and thus largely independent of the charge distribution on the protein surface. The degree of solvent exposure of Trp residues at different pH values can be probed with acrylamide and directly correlated to the structural changes in α-La. Quenching efficiency, presented as the dynamic quenching constant (K_D), is then expected to increase as pH is lowered. Furthermore, interactions between ions and α-La at different pH values are examined in this way, which is presented in Figs. 2 and 3 for Cu^{2+}-ions and Na^{+}-ions, respectively.

In general, quenching efficiency increases markedly with decreasing pH. When first considering the condition without ions applied, lack of Ca^{2+} present implies an overall increase in quenching efficiency. In the presence of Ca^{2+} however, K_D display a stronger pH dependence, with pH 3.5 displaying much lower Trp availability than when Ca^{2+} is not present. Next, addition of $CuCl_2$ and especially $CuSO_4$ decrease Trp availability. Addition of $Cu_3(PO_4)_2$ imply increased quench-

Table 1 Computed T_m values and enthalpies corresponding to each unfolding curve presented in Fig. 1

	pH 2.9		pH 3.3	
	w/o Ca^{2+}	w Ca^{2+}	w/o Ca^{2+}	w Ca^{2+}
T_m (°C)				
w/o ions	27 ± 0.4	34 ± 0.4	34 ± 0.9	40 ± 0.2
$CuCl_2$	26 ± 0.3	30 ± 0.4	31 ± 0.2	33 ± 0.5
$CuSO_4$	20 ± 0.6	27 ± 1	23 ± 0.6	31 ± 0.5
ΔH (kcal/mol)				
w/o ions	24 ± 1	21 ± 1	22 ± 1	28 ± 1
$CuCl_2$	28 ± 1	22 ± 1	34 ± 1	45 ± 3
$CuSO_4$	43 ± 5	20 ± 1	38 ± 4	84 ± 14

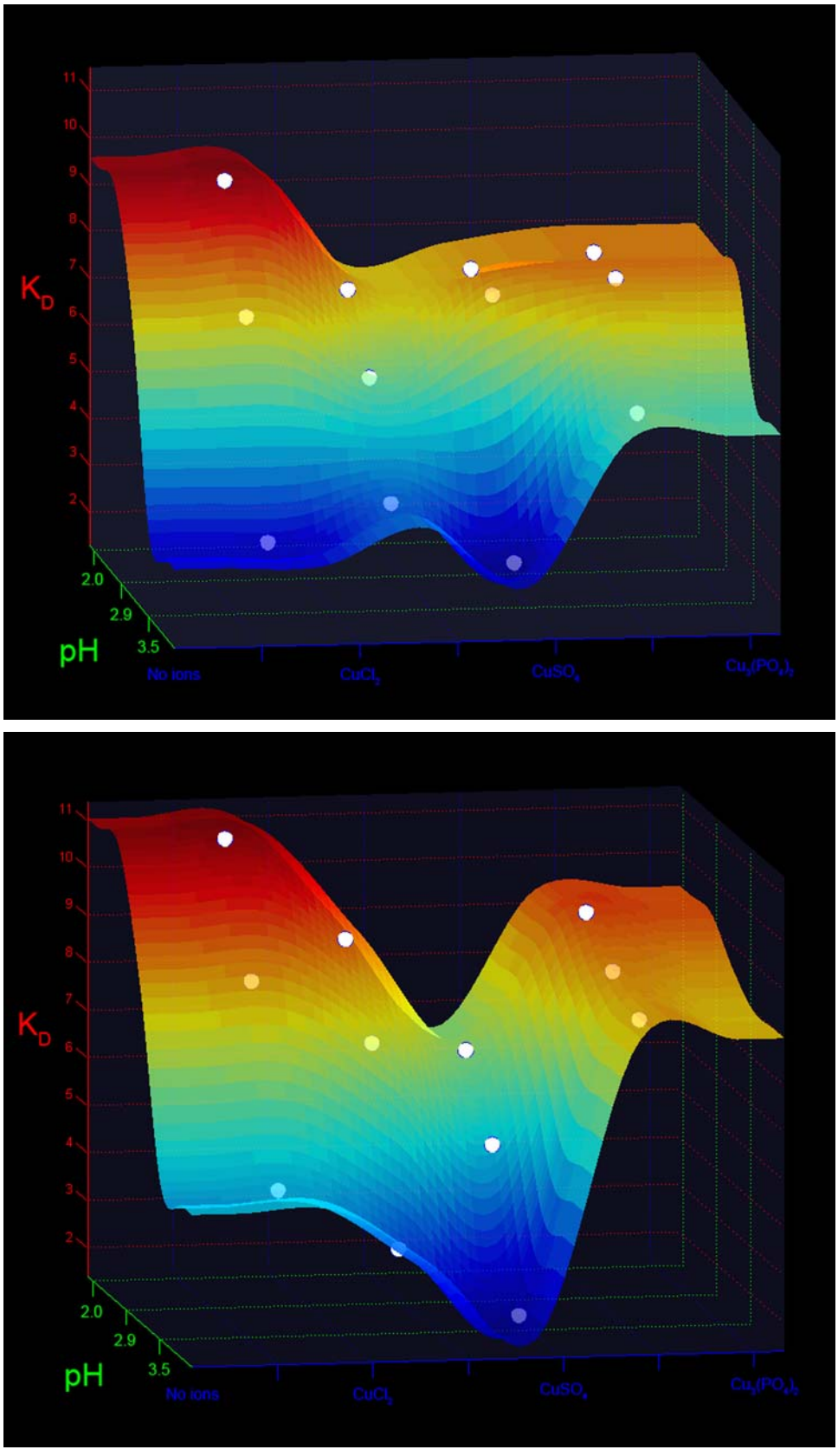

Fig. 2 Quenching efficiency of α-La at different pH and Cu^{2+}-ion combinations; without Ca^{2+} present (*upper*) and with Ca^{2+} present (*lower*)

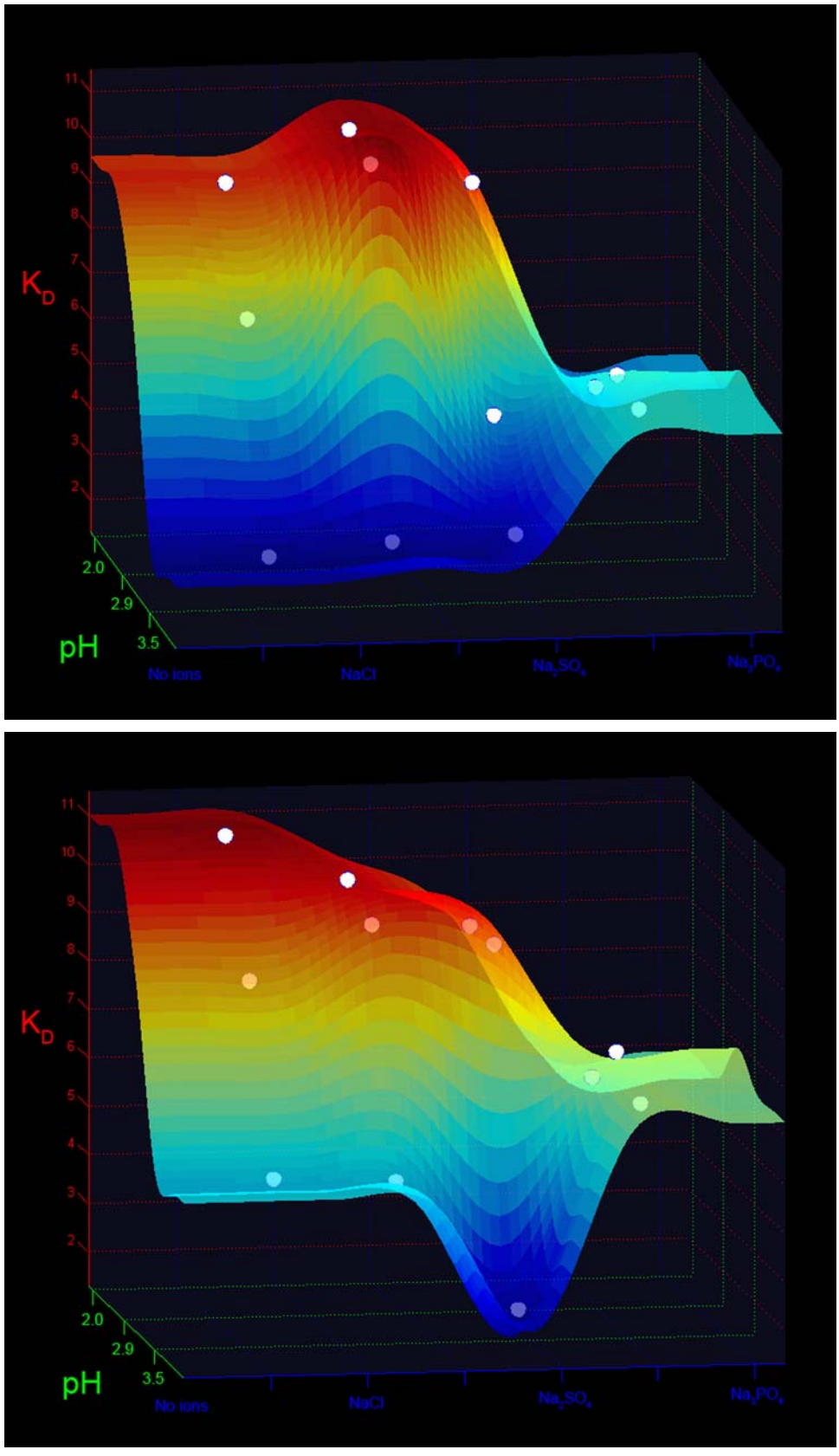

Fig. 3 Quenching efficiency of α-La at different pH and Na^+-ion combinations; without Ca^{2+} present (*upper*) and with Ca^{2+} present (*lower*)

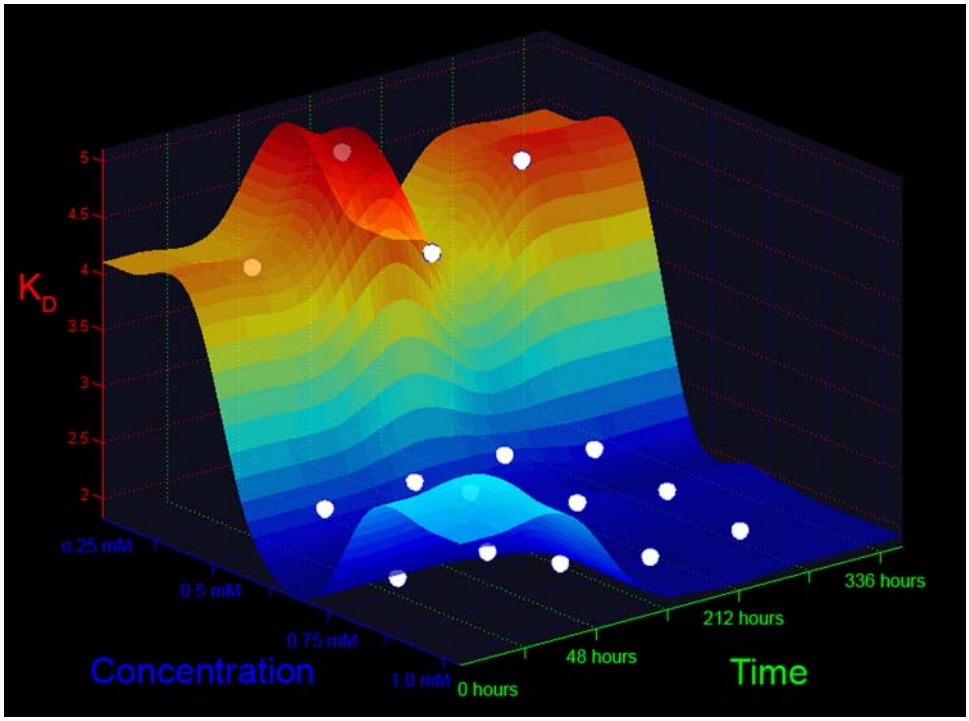

Fig. 4 Quenching efficiency of different concentrations of α-La at pH 3.0 without Ca^{2+} present at different time intervals, incubation at 30°C

ing efficiency. Presence of Ca^{2+} alters the ionic effects on α-La, especially the response to $CuCl_2$. As expected, Na^{+}-ions does not influence the increasing quenching efficiency with decreasing pH. However, the presence of Ca^{2+} exhibits a considerable effect on the interaction of NaCl and Na_2SO_4 with α-La, the effects being both more pronounced and more dependent on pH than what was observed with Cu^{2+}-ions. In correspondence with results obtained by Cu^{2+} ions, addition of Na_3PO_4 imply an increase in solvent accessibility of Trp, however displaying less variance in K_D with pH.

In order to obtain information on the effect of α-La concentration on protein aggregation, an acrylamide quenching experiment has been performed at pH 3.0 in the absence of Ca^{2+}. Hereby, higher protein concentrations are expected to increase the probability and hence rate of aggregation, see Fig. 4. The experiment was repeated at different time intervals to observe the effect of time on α-La aggregation at the different concentrations.

Protein concentration significantly influences quenching efficiency, with concentrations above 0.25 mM resulting in a reduction of solvent accessibility of Trp residues for quenching by acrylamide. Apparently, quenching efficiency remains fairly unchanged over time, although the lowest concentration displays some time dependency.

Confocal laser scanning microscopy

Experiments of CLSM is performed to obtain an optical interpretation of the interactions between Cu^{2+}-ions and α-La. Hereby, all ions are expected to have an impact on α-La aggregation, with $CuSO_4$ inducing most aggregation, see Fig. 5.

As observed, α-La forms aggregates at pH 3.0 even without ions applied. As expected, addition of ions distinctly enhances aggregation of α-La, with the degree depending on the ion type. Hereby, $CuSO_4$ induces more severe amount of aggregation compared to both $CuCl_2$ and $Cu_3(PO_4)_2$. Furthermore, transparent aggregates are formed at the conditions when no ions are present and when $CuCl_2$ is present, whereas the presence of $CuSO_4$ and especially $Cu_3(PO_4)_2$ induces more dense aggregate structures.

Computational titration of α-La

The distribution of charged residues in α-La at acidic pH values is determined by Titra, which is presented in Fig. 6.

As pH is lowered towards acidic values the acidic amino acids become protonated leaving α-La positively charged. The titration of the Asp and Glu residues occurs especially between 4.5 and 3.5.

Discussion

pH effects

According to the thermal stability data, α-La exhibits a thermal unfolding below 40°C, when pH is below pI. The continuous decrease in fluorescence intensity as a function of

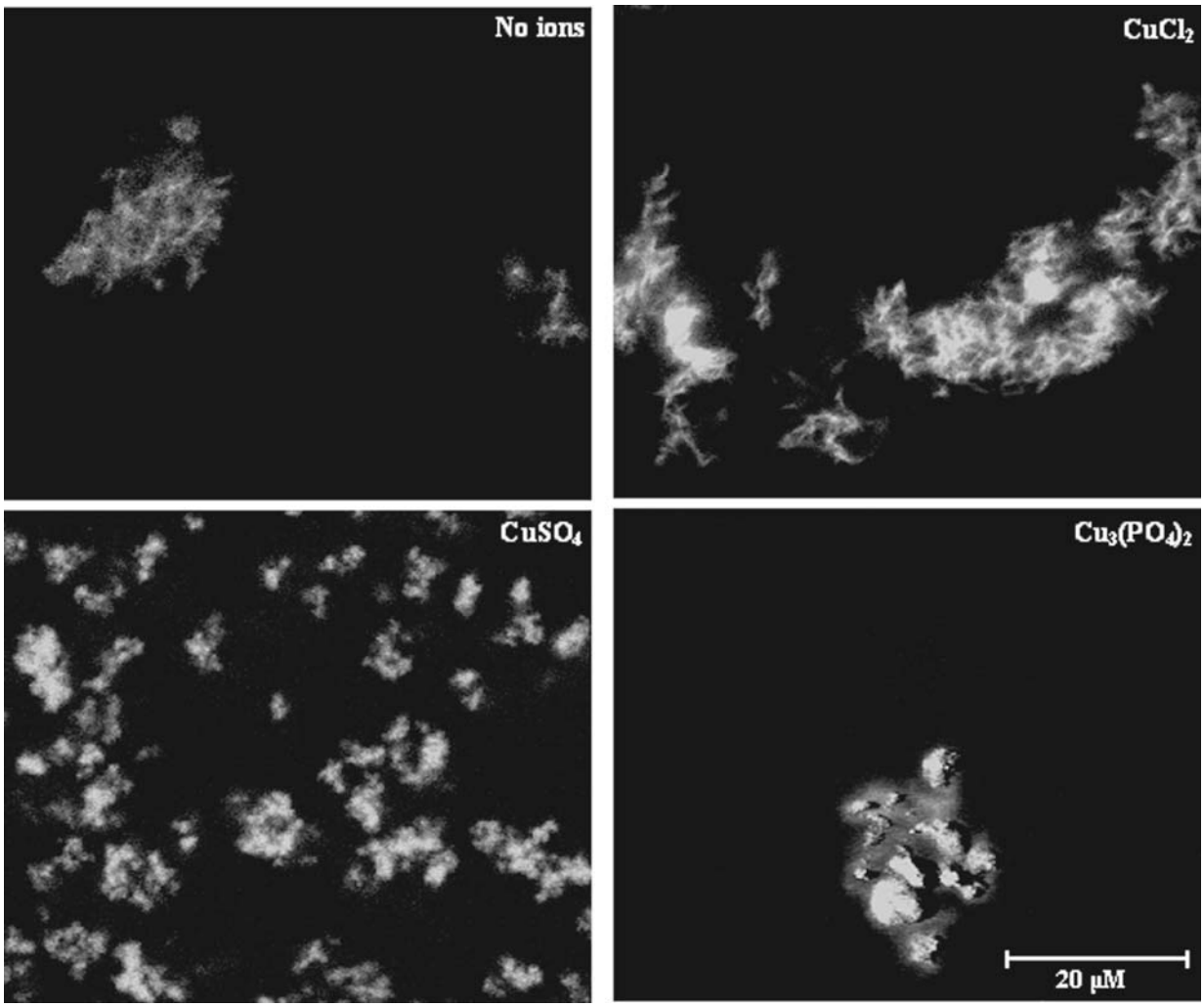

Fig. 5 Confocal laser scanning microscopy images of α-La at pH 3.0 without Ca^{2+} stained with Thioflavin T; without ions present and with $CuCl_2$, $CuSO_4$ and $Cu_3(PO_4)_2$ present. The highlighted bar represents 20 μm

temperature is assigned to the dynamic quenching of Trp residues by water molecules. Thus at unchanged or increase in fluorescence intensity versus temperature is due to increased fluorescence yield of Trp residues. This results from Trp that move from a quenched state in the folded protein to a lesser quenched state in the unfolded protein, overcoming the quenching contribution from water.

The pH responsible destabilisation of α-La can be ascribed to the discharge of Ca^{2+} from the binding pocket as pH is lowered towards acidic values. The resulting molten globule structure exhibit a higher exposure of the hydrophobic interior. As observed by fluorescence spectroscopy, the molten globule will display a red-shifted Trp fluorescence emission and a significantly enhanced fluorescence intensity (data not shown) [18, 19]. The molten globule state leaves α-La structurally destabilised and highly susceptible for aggregation, reflected by a T_m value below 30°C and reduced fluorescence yield during thermal unfolding. The protonation of Asp and Glu residues leads to breakage of salt bridges and thereby a more flexible protein structure with less constraints, and concomitant to rupture of stabilising H-bonds. Hence, as pH is further lowered towards more acidic environment, the structure loss of α-La becomes more pronounced, reflected in T_m values as low as 20–30°C. These observations are further supported by the acrylamide quenching experiments, in which quenching efficiency increases considerably with decreasing pH. This is explained by the less compact structure of α-La rendering Trp residues more available for quenching by acrylamide.

The presented T_m values are somewhat lower compared to previously determined T_m values (32°C at pH 2.5) [11]. This is due to the thermal unfolding experiment being performed at a higher protein concentration, which adds an additional destabilisation factor to the system. In comparison, the concentration of 0.6 mM α-La used in the thermal unfolding experiment corresponds to approximately one half of what

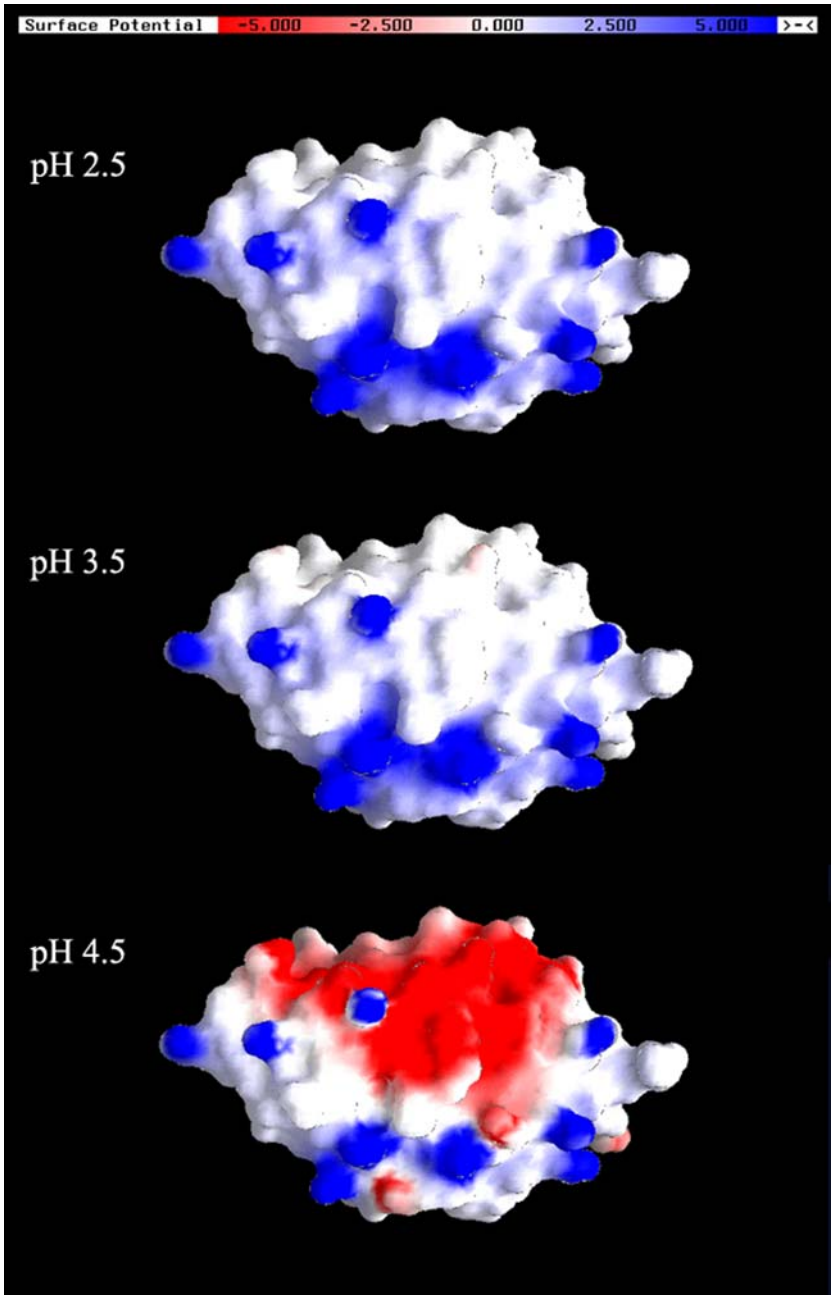

Fig. 6 Computational titration of α-La between pH 2 and 4. The titration was performed without including Ca^{2+} presence. The highlighted amino acids are: basic amino acids (*blue*) and acidic amino acids (*red*)

is applied in a protein beverage. The physical/chemical conditions in acidic protein beverages are precautiously close to T_m for α-La, and thereby facilitating the onset of aggregation.

In the present paper we have shown that α-La forms aggregates solely due to acidic pH, which is evident from results obtained by both confocal microscopy and acrylamide quenching. The molten globule structure is therefore sufficiently unstable to induce aggregation of α-La. Hereby, aggregates are formed at only 300 μM α-La pH 3.0, corresponding approximately to one fourth of the α-La concentration present in protein beverages. It is very likely that α-La is the key responsible protein for aggregation of whey proteins at acidic pH. Evidently, the rate of aggregation then depends on the protein concentration and temperature, whereas specific changes in pH most likely affect the type of aggregates formed due to variations in electrostatic interactions between the proteins.

Ca^{2+} effects

That Ca^{2+} is capable of binding and stabilising the protein at pH below pI is apparent both from the thermal unfolding experiments and the computed T_m values and enthalpies. This is furthermore supported by Trp fluorescence emission

measurements, in which the spectrum is blue-shifted and fluorescence intensity decreased upon Ca^{2+} presence (data not shown). In addition, lack of Ca^{2+} implies an overall increase in acrylamide quenching efficiency, which is correlated with the conformational changes accompanied with the molten globule structure in the absence of Ca^{2+}.

The pH value will influence both Ca^{2+} binding to α-La as well as Trp solvent accessibility. Thus, presence of Ca^{2+} therefore exhibits more stabilisation on α-La at pH 3.3 than 2.9, explaining the higher enthalpy, T_m value and more change in fluorescence yield during thermal unfolding upon Ca^{2+} presence at pH 3.3. This is additionally supported by the quenching experiments, in which K_D display stronger pH dependence in the presence of Ca^{2+}, with pH 3.5 displaying much lower Trp availability than when Ca^{2+} is not present.

Surprisingly, Ca^{2+} exhibits a thermally stabilising effect on α-La even at pH 2.9. One possible interpretation is that Ca^{2+} binding to α-La will result in altered pK_a values of Asp and Glu residues nearby the Ca^{2+}-binding pocket. Concurringly, the pH of Ca^{2+} ejection from α-La is significantly reduced, when Ca^{2+} is present at near neutral pH, compared to when Ca^{2+} is present at pH 3.5 (data not shown). This supports the influence of Ca^{2+} binding on pK_a values of nearby Asp and Glu residues. An alternative explanation could include an interaction of $CaCl_2$ with the surrounding water network rather than residues on the protein surface, thereby favouring the folded conformation of α-La.

Ionic effects

All anions examined destabilise α-La at pH below pI, as expected. According to data obtained by thermal unfolding, acrylamide quenching and confocal microscopy the degree of destabilisation is suggested to be $Cl^- < H_2PO_4^- < SO_4^{2-}$, concurrent with the Hofmeister series [20]. The effect of ions on proteins is usually described by either a preferred interaction with the amino acids on the protein (i.e. preferential binding) or a preferred interaction with the surrounding water network (i.e. preferential hydration), leading to stabilisation of the unfolded or folded conformation of the protein, respectively [20]. Hereby, SO_4^{2-} leads to preferential binding, whereas Cl^- leads to preferential hydration of α-La. Thus, SO_4^{2-} is most efficient in screening the positive charged on α-La, thereby removing the net repulsion between the proteins and promoting random aggregation. In thermal unfolding experiments, the ionic destabilisation is reflected in a lower T_m value and reduced transition during unfolding. When observing quenching efficiency, the destabilisation is correlated with significantly decreased Trp availability upon addition of $CuCl_2$ and especially $CuSO_4$. This is suggesting an ion responsible aggregation of α-La, leading Trp residues inaccessible for quenching by acrylamide. The more pronounced destabilisation by $CuSO_4$ is additionally verified from both considerably increased intensity in static scattered light and altered Trp distribution to emitting maximally at 350 nm (data not shown). The specific action of $H_2PO_4^-$ is somewhat uncertain. According to the results obtained by acrylamide quenching, addition of $Cu_3(PO_4)_2$ imply increased quenching efficiency compared to both $CuCl_2$ and $CuSO_4$, indicating a different interaction of $H_2PO_4^-$ with α-La. This ion specific interaction with α-La most likely provides an explanation to the different induced aggregational structures of α-La, as observed by confocal microscopy. Hereby, both $H_2PO_4^-$ and SO_4^{2-} promote dense aggregate structures, with SO_4^{2-} inducing the highest amount of aggregation, in correlation with the presented results.

Evidently, the interaction of ions with α-La is highly influenced by the specific pH value, since titration of charged amino acid residues influences the interactions between the protein surface and surrounding ion molecules. Hence, less SO_4^{2-}, $H_2PO_4^-$ and Cl^- molecules are required to screen all the positives charges on α-La at pH 3.3 than 2.9, explaining the more pronounced ionic destabilisation observed at pH 3.3. This pH dependency of ionic influence is supported by the computational titration, acrylamide quenching, Trp distribution (data not shown) and static light scattering measurements (data not shown). Conversely, more anions are required to screen α-La as pH is lowered, and the proteins are therefore more stable towards ion caused aggregation at acidic pH values far from pI.

Cu^{2+} is capable of binding to α-La, supposedly to the Zn^{2+}- binding site, which markedly destabilises the protein [21, 22]. The destabilisation of Zn^{2+} binding has been described due to exclusion of Ca^{2+} from the protein. Although Ca^{2+} and Cu^{2+} have been stated to bind concurrently α-La, the specific cause of destabilisation by Cu^{2+} binding still remains to be discovered [21, 22]. Nevertheless, the destabilising effects of $CuCl_2$ on thermal stability of α-La is ascribed to the Cu^{2+}-ion, since a similar experiment with NaCl did not influence the T_m value of the protein significantly (data not shown). It is therefore expected that presence of Cu^{2+} influence the aggregational behavior of α-La. The binding of Cu^{2+} to α-La is supported by the computed enthalpies, in which an overall increase in enthalpy is observed upon addition of Cu^{2+}-ions. This could be explained by Cu^{2+} interacting with the unfolded state of the protein, thereby favouring unfolding of α-La, which explains the concurrent reduction in T_m. Evidently, the binding of Cu^{2+} is pH dependent, explaining the higher enthalpy at pH 3.3 compared to pH 2.9. At pH 2.9 the enthalpy is unaffected by Cu^{2+} addition when Ca^{2+} is present. This suggests that Ca^{2+} is capable of binding to α-La at lower pH values, than Cu^{2+}, thereby stabilising the protein. As previously described, SO_4^{2-} leads to destabilisation of α-La and is therefore likely to facilitate binding of Cu^{2+} ions, explaining the cumulative

destabilisation observed in the presence of $CuSO_4$. Furthermore, SO_4^{2-} is capable of complex formation with Ca^{2+} at pH 3.3, which could serve as an explanation to significantly increase in enthalpy when Ca^{2+} is present.

The destabilisation by Cu^{2+} explains the differences observed in quenching efficiency between Na^+-ions and Cu^{2+}-ions present. The true effects of the anions can then be prescribed to the quenching efficiency observed with Na^+-ions. This furthermore explains the more pronounced effect of Ca^{2+} presence on quenching efficiency observed with Na^+-ions. As observed, especially the NaCl effects are significantly influenced by Ca^{2+} presence. At pH 2.0 the increased Trp availability is most likely due to surplus of Cl^- ions from the $CaCl_2$ rather than Ca^{2+} binding, which would anticipate a reduced Trp availability. One possible explanation could be different aggregate structures induced at the different pH values. Hence, at pH 2.0 NaCl most likely induce fibrillation of α-La, rendering Trp residues more solvent accessible for quenching by acrylamide due to the "pearls on a string" composition of fibrils. Moreover, fibrillation itself might induce conformational changes of the proteins involved. Hereby, particularly fibrillation has been associated with formation of extensive β-structure [23]. Conversely, a more random aggregation is present as pH approaches pI, thereby leaving Trp residues inaccessible to acrylamide. Naturally, additional microscopy experiments are required to state this explicitly. Accordingly the gel appearance of whey proteins is affected by the combination of ions and pH. At pH above or below pI and low ion concentration, heat induced gelation of whey proteins have been described as linear, fine stranded, translucent gels [24, 25]. At higher ion concentration and/or as pH approaches pI, the electrostatic repulsion between protein molecules is reduced, leading to randomly formed aggregates, large enough to scatter the light, yielding opaque gels [26].

Concentration and time effects

According to the results obtained by acrylamide quenching, protein concentrations above 0.25 mM display significant less quenching of Trp residues. This could be due to early stages of aggregation. Moreover, the Stern-Volmer plots of 0.5–1 mM α-La display a deviation from linearity towards the x-axis at higher acrylamide concentrations. This indicates that not all Trp residues are available for quenching, supporting the hypothesis of aggregation (data not shown). In addition, this verifies that the amount of acrylamide is in excess at all proteins concentrations and thus not limiting the experiments. At the concentrations and pH investigated, no significant temporal effects were observed, implying that the aggregation occurs instantaneously. This could though be verified by microscopy experiments. The experiment was conducted at 30°C, which enhances the rate of aggregation. When performing the same experiment below T_m, it is likely that the aggregation information may not be instantaneous.

Evaluation of the quenching experiment

It should be stated that the quenching results presented in this paper are graphically fitted using matlab. Since the dataset is limited, no efforts have been made to pinpoint specific conditions in a multidimensional analysis. However, the 3D graphs highlight strong dependency of quenching on experimental conditions.

Conclusion

Control of aggregation

It is possible to induce aggregation of α-La by depleting the protein of Ca^{2+} and lowering pH, which are the conditions presents in many protein beverages. α-La may therefore play an important role in the aggregation behavior of whey proteins at acidic pH. Stabilisation of the whey protein solutions by maintaining a low protein concentration and a low temperature is not feasible in protein beverages. Thus additional approaches are required to prevent the formation of aggregates in the protein solution. The optimal pH of the solution depends on which ions are present. The lower the pH the less structure of α-La, but also the more stable towards anion promoted aggregation. Hence, addition of Ca^{2+} and strict control of the other ions present is an apparent option. Addition of a protein stabilising agent, such as polyols, could be considered to prevent protein aggregation. α-La has previously been described capable of interacting with membrane systems [27]. Therefore, interaction between α-La and lipid molecules, such as glycerol, phospholipids or fatty acids is likely to stabilise the molten globule state of α-La. The authors believe that the data presented provides novel and potentially important new insight into the complex aggregational behaviour of whey proteins, thus permitting rational design of aggregation in solutions of whey proteins.

Acknowledgements Special thanks are regarded to John Sørensen, Hans Bertelsen and Kristian Albertsen from Arla Foods amba for the invaluable collaboration and constructive discussions. Furthermore, Poul Larsen from Aalborg University is acknowledged for his help in obtaining the confocal microscopy results.

References

1. de Wit JN (1989) The use of whey protein products. In: Developments on dairy chemistry, Elsevier Applied Science, London, pp 323–345
2. Barbut S (1995) Effect of sodium level on the microstructure and texture of whey protein isolate gels. Food Res Int 28(5):437–443

3. Lowe LL, Foegeding EA, Daubert CR (2003) Rheological properties of fine-stranded whey protein isolate gels. Food Hydrocol 17:515–522
4. Rojas SA et al (1997) Gelation of commercial fractions of beta-lactoglobulin and alpha-lactalbumin. Int Dairy J 7(1):79–85
5. Schokker EP, Singh H, Creamer LK (2000) Heat-induced aggregation of β-lactoglobulin A and B with α-lactalbumin. Int Dairy J 10:843–853
6. Shimada K, Cheftel JC (1988) Texture characteristics, protein solubility, and sulfhydryl group/disulfide bond contents of heat-induced gels of whey protein isolate. J Agric Food Chem 36:1018–1025
7. Permyakov EA, Berliner LJ (2000) alpha-Lactalbumin: structure and function. FEBS Lett 473(3):269–274
8. Hill RL, Brew K (1975) Lactose synthease. In: Meister A (ed) Advances in enzymology, Wiley, pp 411–484
9. Dolgikh DA et al (1981) Alpha-Lactalbumin: compact state with fluctuating tertiary structure? FEBS Lett 136(2):311–315
10. Kuwajima K (1996) The molten globule state of α-Lactalbumin. FASEB J 10:102–109
11. Pfeil W (1998) Protein stability and folding. A collection of thermodynamic data. Springer, Berlin Heidelberg New York
12. Håkansson A et al (1995) Apoptosis induced by a human milk protein. Proc Natl Acad Sci USA 92:8064–8068
13. Svensson M et al (1999) Molecular characterisation of α-Lactalbumin folding variants that induce apoptosis in tumor cells. J Biol Chem 274(10):6388–6396
14. Clarke J, Fersht AR (1993) Engineered disulfide bonds as probes of the folding pathway of barnase: Increasing the stability of proteins against the rate of denaturation. Biochemistry 32:4322–4329
15. Lakowicz JR (1999) Principles of fluorescence spectroscopy, 2nd ed. Kluwer/Plenum Publisher
16. Ipsen R, Otte J, Qvist KB (2001) Molecular self-assembly of partially hydrolysed alpha-lactalbumin resulting in strong gels with a novel microstructure. J Dairy Res 68(2):277–286
17. Petersen MTN, Fojan P, Pedersen S (2001) How do lipases and esterases work: The electrostatic contribution. J Biotechnol 85:115–147
18. Permyakov EA, Morozova LA, Burstein EA (1985) Cation binding effects on the pH, thermal and urea denaturation transitions in alpha-lactalbumin. Biophys Chem 21(1):21–31
19. Permyakov EA et al (1981) Calcium-binding to alpha-lactalbumin – structural rearrangement and association constant evaluation by means of intrinsic protein fluorescence changes. Biochem Biophys Res Commun 100(1):191–197
20. Creighton TE (1993) Proteins, structure and molecular properties, 2nd ed. W.H. Freshman and Company
21. Tieghem E, Van Dael H, Van Cauwelaert F (1991) A circular dichroic study of Cu(II) binding to bovine α-Lactalbumin. J Inorg Biochem 42:119–131
22. Van Dael H et al (1992) Conformational aspects of the Cu^{2+} binding to α-lactalbumin. Characterization and stability of the Cu-bound state. Biophys Chem 42:235–242
23. Goers J et al (2002) Conformational prerequisites for alpha-lactalbumin fibrillation. Biochemistry 41(41):12546–12551
24. Doi E (1993) Gels and gelling of globular proteins. Trends Food Sci Technol 4:1–5
25. McClements D, Keogh M (1995) Physical properties of cold-setting gels formed from heat -denatured whey protein isolate. J Sci Food Agric 69:7–14
26. Bryant CM, McClements DJ (1998) Molecular basis of protein functionality with special consideration of cold-set gels derived from heat-denatured whey. Trends Food Sci Technol 9:143–151
27. Cawthern KM, Permyakov E, Berliner LJ (1996) Membrane-bound states of alpha-lactalbumin: Implications for protein stability and conformation. Protein Sci 5(7):1394–1405