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0-Structural Study of κ-casein During Amyloid Formation at Different Temperature

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Abstract: κ-Casein is a milk protein with a unique pattern of disulfide binding. The protein exhibits varying molecular sizes, ranging from monomer to octamer and above in the absence of a reducing agent. This study investigates the fibril-forming propensities and polymerization of reduced κ-casein at temperatures between 25 and 48ºC, by Thioflavin T binding assay, Trp fluorescence intensity, Transmission electron microscopy (TEM) CD spectroscopy and HPLC. ThT and TEM data demonstrate that reduced κ-casein readily forms amyloid fibrils following reduction of its disulfide bonds which increase with increasing temperature. Our findings suggest that reducing agents and temperature induce fibril formation of κ-casein possibly by raising the monomeric state of κ-casein and accelerating its interaction with other κ-casein molecules and eventually forming fibrils. This is also shown by intrinsic fluorescence experiments on reduced κ-casein which reveal that temperature causes an increase in the polarity of the environment of the tryptophans residue. CD spectroscopy also revealed that temperature caused change in the tertiary structure of the protein while the secondary structure showed no considerable change consistent with the hypothesis that the core structure of κ-casein is stable. Consistent with this HPLC data, it can be seen that increasing temperature increases aggregation of reduced κ-casein.

Key word: Amyloid, Aggregation, Polymerization.

INTRODUCTION

Bovine milk contains 3.0-3.5% (w:w) protein, of which 80% is casein (Bobe, G., 1998). The bovine caseins exist in milk as a unique colloidal complex containing inorganic phosphate and calcium, termed the casein micelle. This colloidal complex is composed of submicelles, consisting of αs_1-, αs_2-, β- and κ-casein molecules (Rasmussen, L.K., 1992; Farrell, H.M., Jr., 2003). Among these molecules, only αs_2-casein and κ-casein contain cysteines, with two residues in each polypeptide chain (Kruif, C.G.D. and R.P.May, 1991). Most studies have been done on the αs_1-, αs_2-, β- and κ-casein molecules as they compose the major part of bovine milk and also due to their nutritional importance (Pirs, S.M., 1997). The κ-casein is a specific milk phosphoglycoprotein which presents only one phosphorserine residue per molecule and represent 10% of bovine milk (Panouille, M., 2004; Bernard, H., 1998). The monomeric form of κ-casein has two cysteine residues, which are joined together by an interchain disulfide bond (Panouille, M., 2004; Rasmussen, L.K., 1994; Fox, P.F. and P.L.H. McSweeney, 2003). These cysteines are also capable of forming polymers by participating in interchain disulfide linkages Cys11-Cys11, Cys11-Cys88 and Cys88-Cys88 (Rasmussen, L.K. 1992; Rasmussen, L.K., 1994; Fox, P.F. and P.L.H. McSweeney, 2003). Thus κ-casein in aqueous solution forms large spherical polymers with an average molecular mass of 1.18 MDa ranging from monomers to decamers. The relatively unstructured nature of the monomeric form of κ-casein may be the reason for its inherent propensity to form amyloids (Rasmussen, L.K., 1992; Groves, M.L., 1998; Vreeman, H.J., 1981).

In some disease such as amyloidoses, in which protein misfolds, protein goes from its native soluble form to insoluble fibril which is highly β-sheet in character forming rope-like assemblies known as amyloid fibrils (Dobson, C.M., 2003; Arrigo, A.P. and W.E.G. Muller, 2001; Chiti, F., 1999; Come, J.H., 1993). Amyloid formation is associated with a number of diseases e.g. Alzheimer's, Parkinson's, Huntington's, Creutzfeldt-Jakob diseases (CJD) and late-onset. Structural studies from the amyloid β-peptide of Alzheimerr's diseases have revealed information about their structure (Tycko, R., 2000; Petkova, A.T., 2002; Wetzel, R., 2002). In addition these amyloid fibrils have been identified in bovine, rat and canine mammary glands known as corpora mammary glands (Reid, I.M., 1972; Beems, R.B., 1978). The proteins involved in amyloid fibril formation

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have not been determined, however several milk proteins including the casein have been suggested (Claudon, C., 1998).

It has been speculated that a reducing agent induces amyloid formation in κ-casein since it causes the monomeric form of it (Thorn, D.C., 2005). In doing so, the aggregation of reduced κ-casein should increase, as the temperature increases. A theoretical energy-minimized three-dimensional model of κ-casein proposed by Kumosinski *et al.* (1991) suggests that the monomeric form of the protein can adopt a "horse and rider" configuration in which the legs of the horse are two sets of antiparallel β-sheets (from Lys^{21} to Phe^{55}), which are rich in hydrophobic side chains. It has been speculated that the putative antiparallel β-sheet legs, due to their high degree of hydrophobicity and exposure to the solution, may facilitate the amyloid fibril formation by κ-casein (Lencki, R., 2007; Farrell, H.M.J., 2002).

Many studies have been made of the heat stability of casein micelles or their modifications at high temperatures, however, very few studies deal with the heat-induced aggregation of casein submicelles (Farrell, H.M., Jr., 2003; Panouille, M., 2004; Singh, H. and P.F. Fox, 1986; Singh, H. and P.F. Fox, 1987; Schmidt, D.G., 1982). We investigated the ability of fibril formation by κ-casein by raising temperature. Having compared the extent of κ-casein fibril formation at different temperatures, it can be seen that the fibril-forming propensity of κ-casein is increase by increasing temperature, however, the secondary structure of protein changed very little.

MATERIAL AND METHODS

κ-Casein, 1,4-dithiothreitol (DTT), sodium azide (NaN_3), sodium hydrogen phosphate (Na_2HPO_4), thioflavin T (ThT) were obtained from Sigma-Aldrich (St. Louis, U.S.A.).

Fibril Formation and Kinetic Evaluation:

Amyloid fibril formation of reduced κ-casein (2.5 mg/mL) at different temperatures (25, 37, 42, 48ºC) were investigated by incubation of protein in a 50 mM phosphate buffer, 20 mM DTT and pH 7.4 in an incubator (A-Q, Germany). To accelerate fibril formation, solutions were shaken at 210 rpm. Fibrillation of κ-casein was monitored using a Varian spectrofluorimeter equipped with a thermostated circulating water bath. Aliquots of 10 μL were sampled and amyloid formation was detected by the increase in ThT fluorescence (0.4 μM in 50 mM Na_2HPO_4, 0.05% (w/v) NaN_3, pH 7.4). The excitation and emission wavelengths were set to 378 nm and 479 nm with 2.5 nm and 5 nm slit widths, respectively.

Intrinsic Fluorescence Spectroscopy:

Intrinsic fluorescence intensity was measured on samples containing natural and reduced κ-casein to investigate the effect of temperature on the environment of the tryptophan residues of κ-casein. Samples containing κ-casein (1 mg/mL) with 20 mM DTT were incubated in a 50 mM phosphate buffer, 0.05% (w/v) NaN_3 and pH 7.4 for 3 hrs at 25, 37, 42, 45 and 48ºC. Control experiments with the same sample components were carried out without the addition of DTT.

Fluorescence spectra were obtained on a varian fluorescence spectrofluorimeter equipped with a thermostated circulating water bath. Tryptophan residues were excited at 295 nm using a 2.5 nm slit width and emission spectra were recorded from 300-400 nm with a 5 nm slit width. The instrument was set to 700 V with a scan speed of 240 nm/min.

Transmission Electron Microscopy (TEM) of κ-casein:

Formvar and carbon-coated nickel TEM grids (SPI Supplies, Wester Chester, USA) were prepared by the application of a 2 μL protein sample at a concentration of 1 mg/mL. The grids were then washed three times with10 μL MilliQ water and negatively stained with 10 μL uranyl acetate (2% w/v; Agar Scientific, UK). The grids were dried with filter paper between each step. The samples were viewed under 25-64 K magnifications at 80 kV excitation voltages using a Philips CM100 transmission electron microscope (Philips, Eindhoven, The Netherlands). The dimensions of the fibrils were estimated by observing the images at 200% zoom in paintbrush (Microsoft Corporation, USA) and by comparing them with the length of the scale bar using ImageJ software.

Circular Dichroism (CD) Spectroscopy of κ-casein:

Far and Near-UV CD experiments were carried out with 0.1 and 0.2 mg/ml protein samples respectively with and without 20 mM DTT in a 10 mM phosphate buffer, pH 7.0. Successive measurements in the far UV (190 to 250 nm) and Near (250-350nm) were made at 25, 37, 42, 45, and 48°C. The measurements were taken

in 1 cm pathlength cuvette using an Aviv spectropolarimeter (Aviv, INC. Model 215) connected to Aviv water bath. Spectra were recorded with a data interval of 1 nm, response time of 2 seconds and a scan rate of 100 nm/min. Each spectrum was an average of 4 scans, with a baseline scan subtracted.

Size Exclusion HPLC of κ-casein at Different Temperature (25, 37, 42, 45 and 48°C):

The size of κ-casein (2.5 mg/mL) aggregates at different temperatures after incubation in a 50 mM phosphate buffer, pH 7.0 were determined by size-exclusion chromatography after injecting 25 µL of the samples. A Zorbax GF-250 Gel Filtration Column, 250 ´ 4.60 mm (Zorbax ®, Agilent, Germany.) with a mobile phase of a 10 mM sodium phosphate buffer, pH 7.2, was used on an Agilent HPLC system by an SLC-10A vp pump system organizer, with a Dual wavelength detector and autosampler injection (Agilent, Germany). This column has a mass range of 900 kDa to 4 kDa for native proteins. The aggregate size of samples calculated using standard curve and compared.

Results:

Investigating the Amyloidogenicity of Reduced κ-Casein:

κ-Casein contains extensive intermolecular disulfide bridging in its native state (Rasmussen, L.K., 1992) and is therefore potentially susceptible to destabilization of its quaternary structure by reducing agents. In this study, κ-casein fibril formation was induced at neutral pH via reduction. An enhancement in thioflavin T fluorescence was used to show the possibility of κ-casein amyloid formation.

Figure 1 shows the time course of ThT fluorescence for reduced κ-casein incubated between 25 and 48°C. Reduced κ-casein displays a substantial capacity to bind ThT, as previously reported (Farrell, H.M., Jr., 2003; Thorn, D., 2005). When the temperature was raised to 48°C, however, a time-dependent increase in fluorescence was observed (Fig. 1). This was also evident from the first-order rate constant which increased with increasing temperature and exhibited no discernible lag phase (Table 1).

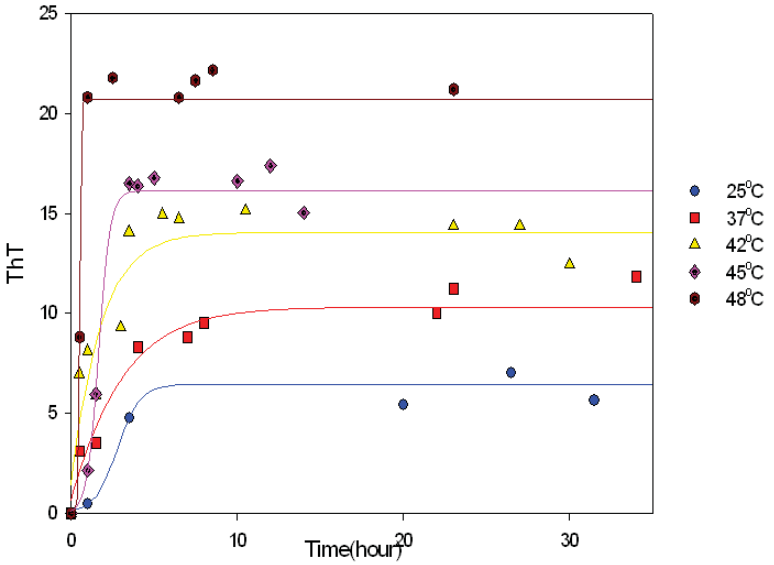


Fig. 1: κ-casein (2.5 mg/mL) amyloid formation at diferrent temperature (25, 37, 40, 45 and 45 °C)**.** Protein was in 50 mM sodium phosphate buffers, 0.05% (w/v) NaN_3; pH 7.4, 4 µM ThT. The amyloid fibril formation of κ-casein was initiated by the addition of 20 mM DTT. The experiment was done twice and fitted using sigmaplot software.

Table 1: Summary of rate constants for -casein, in thioflavinT binding assays. The rate constants were calculated by fitting exponentional function to thioflavinT T binding data using Sigmaplot software.

κ-casein (°C)	Rate constant (min^{-1})
25	$(1.44\pm0.45) \times 10^{-4}$
37	$(3.59\pm0.49) \times 10^{-1}$
40	$(5.87\pm1.7) \times 10^{-1}$
45	2.81±0.15
48	5.31±0.12

Analysis by TEM at 37 and 45°C confirmed the formation of fibrils by κ-casein at increasing temperatures. At 37°C temperature in the presence of DTT for 72 h, κ-casein formed threadlike structures, approximately 12-16 nm in diameter and 50-300 nm in length (Fig. 2A). When the temperature was increased to 45°C DTT, these fibrillar species were noticeably longerr (more than 200 nm; Figure 2B), consistent with the observed differences in ThT binding (Fig. 1).

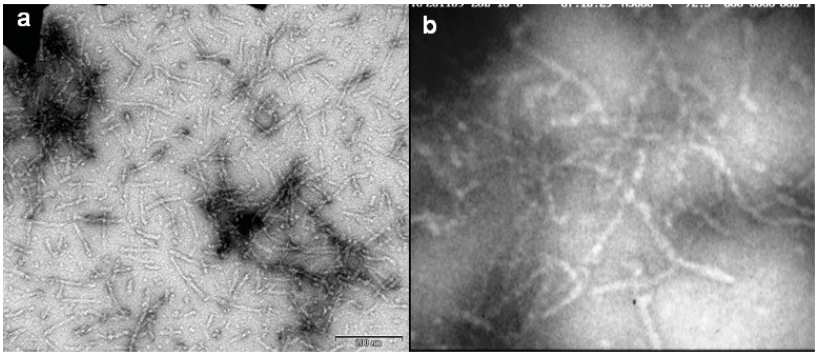


Fig. 2: Negatively stained electron micrograph of reduced κ-casein at (A):37ºC (B):45ºC. κ-Casein (2.5 mg/mL) was incubated in 50 mM phosphate buffer, pH 7.5 and 20 mM DTT for 72 hrs, before being analyzed by TEM. Scale bars represent 200 nm. Note that the average length of fibril was calculated by measuring all fibrils within a selected representative area using Image J software.

Intrinsic Tryptophan Fluorescence Spectroscopy of κ-casein:

Further investigations of the effect of temperature on the structure of κ-casein were undertaken via measurements of the intrinsic fluorescence of κ-casein. Figure 3 shows a comparison of maximum fluorescence wavelength (λ_{max}) and fluorescence intensity at λ_{max} of native and reduced κ-casein between 25 to 48ºC. κ-Casein showed a high fluorescence emission from its single tryptophan residue at position 76 (10). After 3 hours of incubation of κ-casein with DTT at 25ºC, the intrinsic tryptophan fluorescence decreased, indicating a conformational change which leads to more solvent exposure of tryptophan. Higher temperatures of 25 to 48ºC promoted further decrease in fluorescence intensity and increase in wavelength. Such results have also been reported with the αA- and αB-crystallin (Liang, J.J., 2000). Our observations imply that DTT have a large effect on the κ-casein. However temperatures have the effect with both natural and reduced κ-casein by more effect on reduced κ-casein.

Investigating of Structural Change of K-casein by Circular Dichroism (CD) Spectroscopy:

Far-UV CD measurements (190–240 nm) of reduced and non-reduced κ-casein, showed spectra with a minimum of ellipticity around 196 nm and a secondary negative between 210 and 230 nm (Fig. 4), similar in their general features to those already observed by others (26,32). Structural predictions from these spectra demonstrate significant amounts of secondary structure as shown in Table 2.

The CD spectra obtained at each of the reduced and non-reduced κ-casein were similar for the heated as well as for the unheated protein. Comparison of such spectra showed that these structures remain quite stable as they are heated and show no significant changes up to 48°C. These observation support the idea that κ-casein may contain a heat-stable core with well defined secondary structures (Farrell, H.M.J., 2002; Paulsson, M. and P. Dejmek., 1990).

Near-UV spectra of reduced and non-reduced κ-casein submitted to different heating treatments can be observed on Figure. 5. In both cases of samples at pH 7.2, the CD signal shows a negative ellipticity at around 272 nm and 275 nm and a positive peak at 290-297 nm due to the nine tyrosine and one tryptophan residues, respectively. A spectrum recorded after increasing the temperature to 37ºC showed a slight increase in the Trp signal at around 299 nm indicating some change in the environment of the single tryptophan residue (Trp 76) correlating with the intrinsic fluorescence results. All recorded spectra showed increase by the time the temperature reached 48ºC. The spectra around the 272 nm showed consistent decrease in the intensity as temperature increased between 25º to 48ºC. This effect could also be caused by the cleavage of the disulfide bonds, as was reported for the reduction of α-lactalbumin in a previous study (Ewbank, J.J. and T.E. Creighton, 1993) indicative of a decrease in the amount of tertiary structure due to unfolding of the protein.

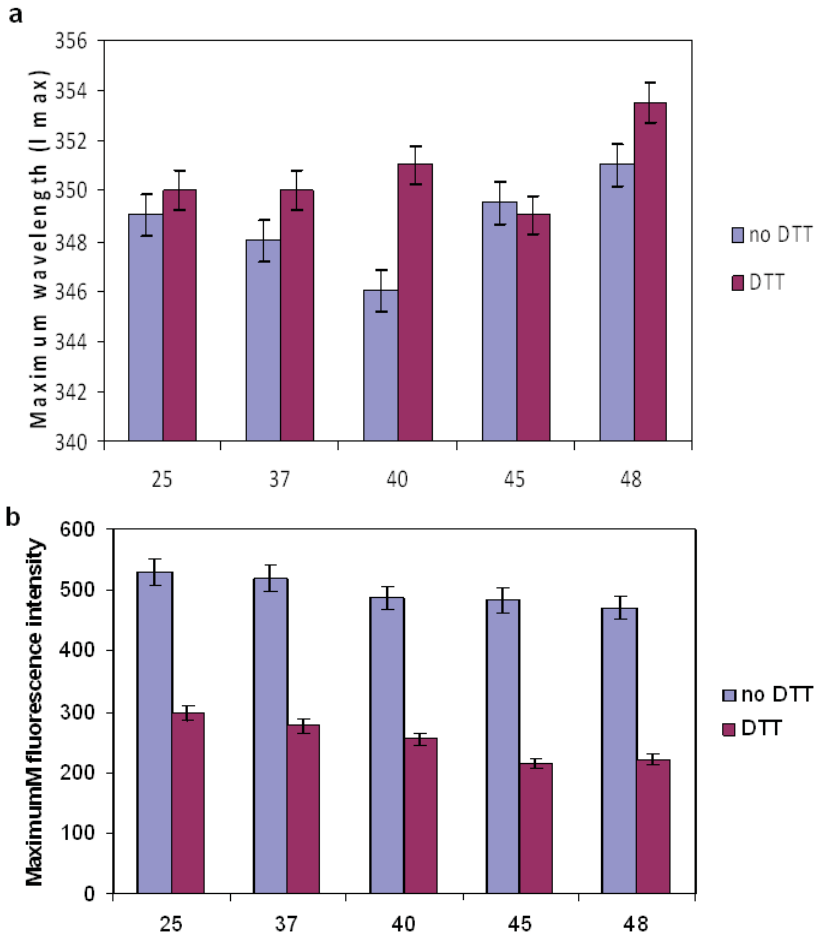
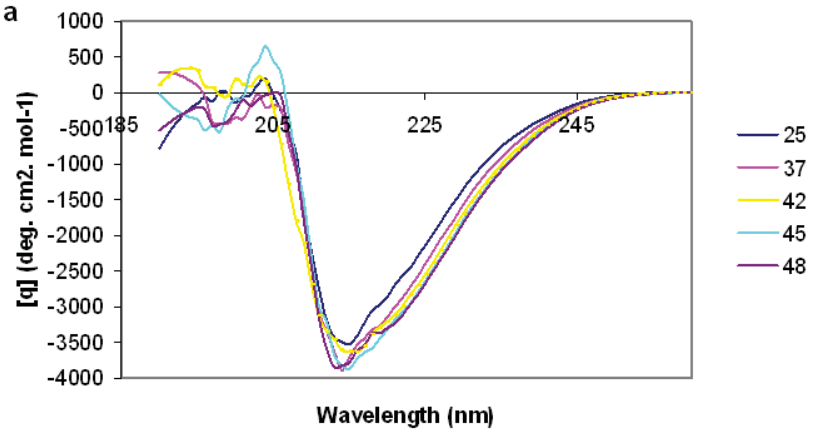


Fig. 3: (A): The maximum intrinsic fluorescence of κ-casein (1 mg/mL) and (B): The average l_{max} of κ-casein (1 mg/mL). All experiments were conducted in a 50 mM phosphate buffer, 0.05% (w/v) NaN_3, pH 7.4 between 25 to 48°C. The error bars are absolute values of maximum calculated errors.



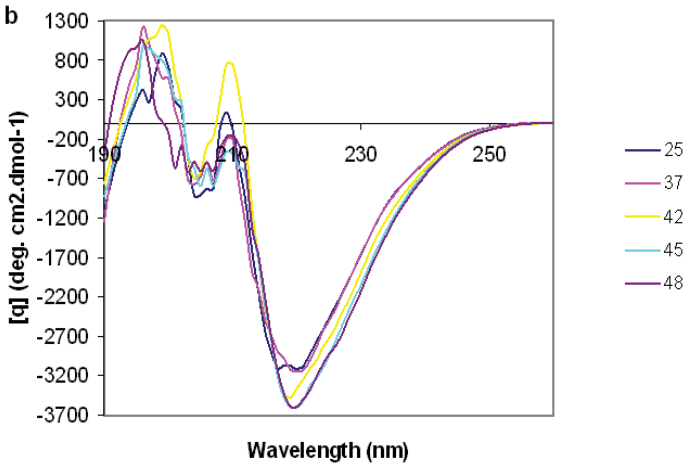


Fig. 4: Far-UV CD spectra of the (A): the reduced state, (B): native state of κ-casein at different temperature (25, 37, 42, 45, and 48ºC). Protein concentration was 1 mg/mL in 10 mM phosphate buffer, pH 7.0, in an Aviv spectropolarimeter with a 1 cm pathlength cell.

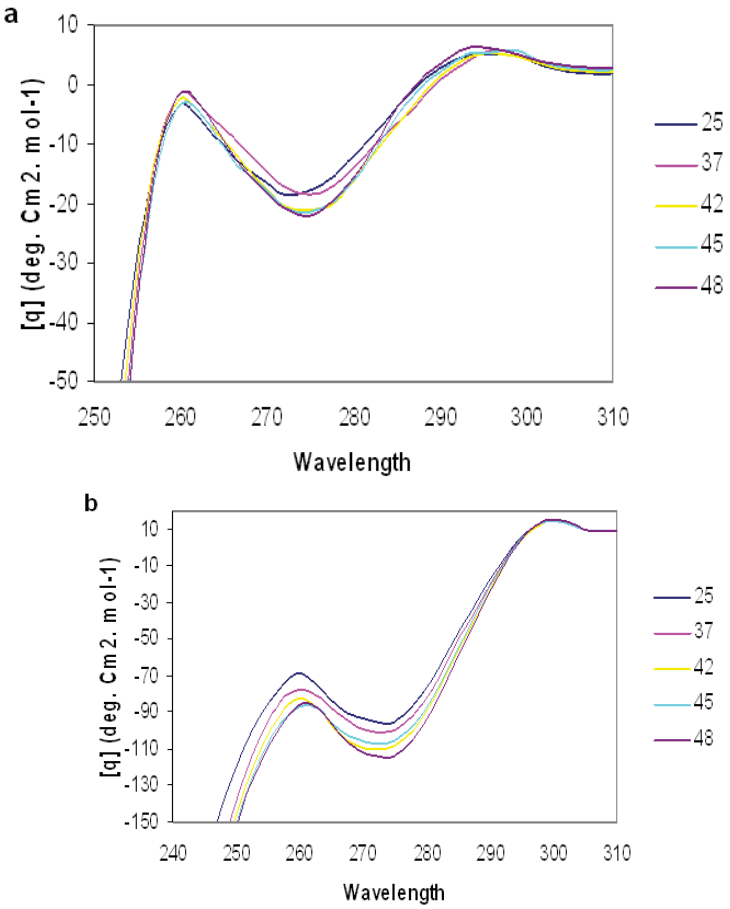


Fig. 5: Near-UV CD spectra of the (A): the reduced state, (B): native state of κ-casein at different temperature (25, 37, 42, 45, and 48ºC). Protein concentration was 2 mg/mL in 10 mM phosphate buffer, pH 7.0, in an Aviv spectropolarimeter with a 1 cm pathlength cell.

Table 2: Secondary structural prediction from Far-CD spectra for the reduced and native -casein using the Aviv program.

	Rduced state			Native state		
Temperature (°C)	α Helix	β sheet	Random	α Helix	β sheet	Random
25	11.9	46.3	41.8	12.4	46	41.6
37	11.6	46.3	42.1	13.1	45.6	41.3
42	12	46.1	41.91	13.1	45.6	41.3
45	11.7	46.3	42	13	45.7	41.3
48	11.7	46.3	42	12.8	45.7	41.5

Size Exclusion HPLC of κ-casein at Different Temperature:

Size exclusion HPLC was employed to investigate and compare the retention time and consequently size of κ-casein molecules at different temperatures.

As shown in Figure 6, all samples of κ-casein loaded onto the column eluted as symmetrical peaks. κ-Casein at 25ºC eluted as a major peak at approximately 5.2 min with an average mass of 24.81 kDa. By increasing temperature up to 48ºC the retention time decreased to 4.66 and eventually the aggregate mass of κ-casein showed an increase (Fig. 7). It also showed peaks around 8 and 9 minutes, which correspond to dimeric or monomeric species.

It is obvious, however, that κ-casein at all temperatures appeared to form aggregates which were larger at the higher temperature. The higher the temperature, the greater the gains of monomeric and dimeric κ-casein species with a concurrent increase in the amount of more highly aggregated species. Thus, higher temperature caused greater aggregation of κ-casein.

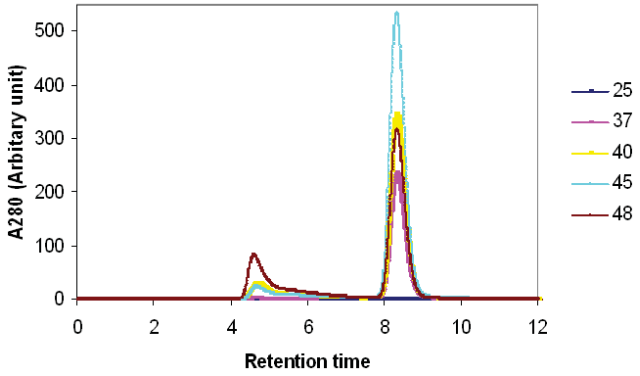


Fig. 6: Size exclusion HPLC traces for κ-casein (2.5 mg/mL) aggregates at different temperatures (25, 37, 42, 45 and 48ºC). 25 µL aliquots of samples in 50 mM sodium phosphate buffer, 0.05% (w/v) NaN_3 and pH 7.4 were injected onto the column.

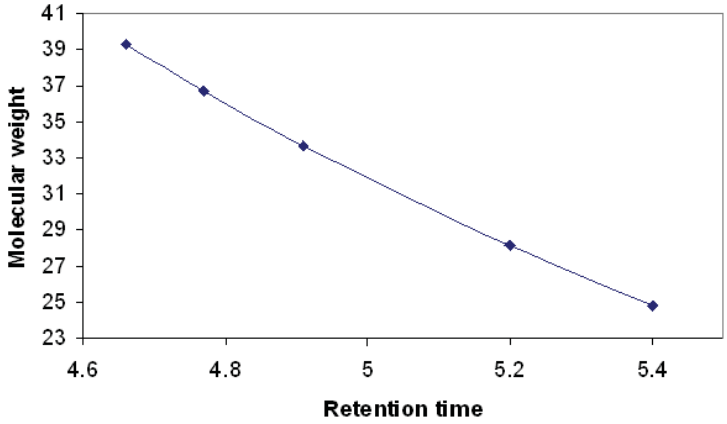


Fig. 7: Average molecular masses (kDa) aggregates of κ-casein around the 5.4-4.6 minute retention at different temperature. Average molecular masses were calculated using the standard curve.

Discussion:

In vitro, κ-casein forms large spherical polymers of ~18 nm in diameter, with an average molecular mass of 1.18 MDa (Groves, M.L., 1998; Farrell, H.M. Jr., 1996), which are assembled from smaller multimeric subunits (monomers to decamers) that result from intermolecular disulfide bonding (Rasmussen, L.K., 1992). When disulfide bond formation is prevented by reduction and/or alkylation, however, such an arrangement is no longer favorable and κ-casein forms long rod-like structures that display many of the characteristics of amyloid fibrils (Farrell, H.M. Jr., 2003; Farrell, H.M. Jr., 2002). Knowing this, we investigated the ability of reduced κ-casein to form amyloid at different temperatures. Fibrillar aggregates of reduced κ-casein at 25ºC are formed and incubation at temperatures of 25ºC to 48ºC induces a conformational rearrangement characterized by an increase in ThT binding (Fig. 1). Consistent with this, TEM examination of reduced κ-casein incubated at 37ºC for 72 hours clearly shows the presence of fibrils of up to 200 nm in length and an increase in the number and length of fibrils by increasing of temperature to 45ºC (Fig. 2).

The intrinsic fluorescence experiments on native and reduced κ-casein at the incubation time of 25 to 48ºC also reveals that temperature causes an increase in the polarity of the environment of the tryptophan residue (position 76) in κ-casein. This increase in polarity is most likely a result of conformational change that either exposed Trp to the surface of the protein and therefore, to the solvent to a greater extend or brought Trp into closer proximity to charged groups on the protein. This may encourage the formation of undesirable contacts with other κ-casein molecules, which in turn increases the self assembly of κ-casein into a fibrillar structure under these conditions.

κ-Casein is classified as an unfolded protein and in its native state exists as a molten globule-like structure with a significant amount of exposed hydrophobic area even in the disulphide-linked polymer (Thorn, D., 2005; Farrell, H.M.J., 2002; Dunker, A.K., 2002). Molecular modeling of κ-casein predicts that the secondary structure of κ-casein implies that it has a considerable amount of β-sheet, which is highly hydrophobic (Kumosinski, T.F., 1993). These hydrophobic areas in κ-casein are ideal sites for sheet-sheet interactions with other κ-casein molecules (Kumosinski, T.F., 1991; Kumosinski, T.F., 1993) as occurs during fibril formation. Pepper *et al*. (1982) report that upon addition of a reducing agent, κ-casein becomes a high molecular mass polydisperse complex, in a concentration-dependent association with itself or with other proteins. By increasing temperature, however, the fluorescence intensity of the reduced κ-casein, was lower than the fluorescence intensity in the absence of a reducing agent. We can conclude, therefore, that temperature decreased the degree of exposed hydrophobicity of κ-casein, possibly by promoting fibrillation. This was also supported by thioflavin T binding and by size exclusion HPLC data, which showed greater aggregation of κ-casein at increasing temperatures. HPLC data and ThT binding of κ-casein showed that temperature increased both the rate and extent of amyloid formation (Figs. 1, 6 and 7). In agreement with these data, Farrell *et al*. (2003) reveal that native κ-casein forms a self-associating polymeric species at 37ºC. RCM-κ-casein (reduced and carboxymethylated) when heated up to 37ºC, however, exhibited an increase in average molecular mass due to self-association and the formation of fibrillar structure. The authors propos that β-sheet-turn-sheet motifs might be responsible for fibril formation. Other studies (Rasmussen, L.K., 1992; Farrell, H.M., Jr.,2003; Groves, M.L., 1998; Vreeman, H.J., 1981) report that κ-casein exhibits four distinct types of self-association and the completely reduced form leads to fibril formation.

Near UV CD spectra of κ-casein also showed differences in the positions and intensity of bands in the spectra in the reduced and non-reduced κ-casein at different temperatures. The effect of DTT and temperature on the tertiary structure of κ-casein and the environment of aromatic and cysteine residues was therefore significant. With the results of tryptophan fluorescence, it appears that temperature causes alteration in the environment of tryptophan residues. Likewise far UV CD spectra shows that temperature has little effect on the secondary structure of κ-casein, which is a naturally unfolded protein.

Conclusion:

As shown by thioflavin T binding assays, temperature increased the rate of amyloid formation by κ-casein. Possible causes of the effect of temperature on the rate and extent of fibril formation of target protein were investigated by thioflavin T binding, intrinsic fluorescence spectroscopy, TEM, and size exclusion HPLC.

Intrinsic fluorescence spectroscopy of κ-casein indicated that temperature increased amyloid fibril formation possibly by raising the monomeric state of κ-casein and accelerating its interaction with other κ-casein molecules and eventually forming fibrils. Temperature caused structural changes in the environment of the tryptophan residues of κ-casein on observation, which was supported by Near UV CD. This may increase the dissociation of the κ-casein and increase its self-assembly consistent with the TEM and HPLC results.

In summary, temperature increases the rate of amyloid aggregation as well as self assembly of κ-casein.

Abbreviation:

Dithiothreitol (DTT), Thioflavin T (ThT), Circular Dichroism (CD), Transmission Electron Microscopy (TEM), High Pressure Liquid Chromatography (HPLC).

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