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RAPID COMMUNICATION

Consequential secondary structure alterations and aggregation during prolonged casein glycation

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Abstract Non-enzymatic glycosylation (glycation) of casein is a process used not just to ameliorate the quality of dairy products but also to increase the shelf life of canned foods, including baby milk supplements. Incubation of κ-casein with reducing sugars for 15 days at physiological temperature showed the formation of a molten globule state at day 9 and 12 during fructation and glucation respectively. This state exhibits substantial secondary structure and maximum ANS binding. Later on, glycation resulted in the formation of aggregates at day 12 in presence of fructose and day 15 in presence of glucose. Aggregates possess extensive β-sheet structure as revealed by far-UV CD and FTIR. These aggregates showed altered tryptophan environment, decrease ANS binding relative to molten globule state and increase in Thioflavin T fluorescence. Aggregates were also accompanied by the accumulation of AGEs, indicative of structural damage to the protein and formation of potentially harmful species at the physiological level. Fructose was more reactive than glucose and thus caused early and significant changes in the protein. From our studies, we conclude that controlling the extent of the Maillard reaction in the food industry is essential to counter its negative effects and expand its safety spectrum.

Keywords Advanced glycation end products · Aggregation · Circular dichroism · Glycation · Maillard reaction · Molten globule state

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Abbreviations

ANS	8-Anilino-1-Naphthalene-Sulphonic acid
CD	Circular dichroism
FTIR	Fourier transform infra-red spectroscopy
MRE	Mean residual ellipticity
Th T	Thioflavin T
MR	Maillard reaction
AGEs	Advanced glycation end products
MDA	Malondialdehyde
FRET	Fluorescence resonance energy transfer
mRNA	messenger RNA
PBS	Phosphate buffer saline

Introduction

Protein folding is the physical process by which a polypeptide, after being translated from a sequence of mRNA to a linear chain of amino acids, folds into its characteristic and functional three-dimensional structure [1]. The information necessary to drive a protein into its native three-dimensional structure under physiological conditions is known to be encoded within its amino acid sequence. However, a protein exists as an intermediate state between its unfolded and native state depending upon its environment, such as pH, temperature, crowding, etc. Under certain conditions, proteins can also exist as a collapsed state with a partial ordered structure known as the "molten globule" [2]. A molten globule has a native like secondary structure with a radius of only 10–20 % larger than that of the native structure but there is absence of specific tertiary packing interactions of the side chains [3]. Further destabilization of protein leads to aggregate formation in competition with the normal

protein folding pathway. Protein aggregates can be of two types based on their solubility: soluble and insoluble aggregates. They can be covalently linked through disulphide bond formation or may be held via weaker interactions as in reversible protein aggregates [4, 5]. Aggregation may occur by reversible association of native protein monomers or by association of modified monomers [6, 7].

Casein products are mainly used as ingredients in foods for either providing nutritional supplements or modifying the physical properties of that food product. One of the most important roles in improving these properties is played by the Maillard reaction (non-enzymatic glycation) that occurs during heating of food. The incubation of proteins especially casein, with reducing sugars to form glyco-conjugates via the Maillard reaction at high temperatures, has been accepted by the industry as one of the most efficient, quickest, and widely used methods. The glycated proteins through the MR are added as functional ingredients into foods. The reaction improves the emulsion, flavor, aroma, appearance as well as texture of the food by enhancing the stability and structure of the proteins present in it. [8]. However, reports suggest that the process also results in the loss of nutritive value and formation of mutagenic compounds, resulting in decreased food safety. In various studies [9–11] this loss was attributed to the destruction and/or biological inactivation of amino acids, including essential amino acids like Lys and Trp, inhibition of proteolytic and glycolytic enzymes, and interaction with metal ions. The reaction mechanism seems to have a major influence in the mutagenicity of the reaction products. Ketose sugars showed a higher mutagenic activity than the corresponding aldose sugars [12]. Fructose, a ketose sugar, exists to a greater extent in the open-chain form than glucose (aldose sugar). Hence the initial stages of the MR occur more rapidly in fructose than glucose, contributing more to the mutagenic compound [13].

Bovine casein consists of four major components: αs1-, β-, κ-, and αs2-caseins. Among these components, κ-casein is of particular interest because it is thought to be responsible for the steric stability of casein micelles through coating of the structure. Owing to the extra stability of κ-casein over other subtypes of casein, we have chosen it to study the effect of glycation as it is very likely that structural impairment of κ-casein will also cause other vulnerable forms (αs1-, β-, αs2-) to suffer damage. It contains two cysteine residues, which give rise to a specific disulphide bonding pattern under non-reducing conditions [14].

The present work is done to investigate the role of reducing sugars i.e. glucose and fructose to induce the MR in κ-casein at physiological temperatures for a prolonged period. Our study indicates that casein not only gets glycated at 37 °C but even results in the formation of aggregates and AGEs at the later stages. This glycated casein, when consumed in the form of processed food, will cause damage by producing mutagenic compounds and cross linked species once they enter the human body.

Experimental

Materials and Methods

κ-casein was purchased commercially from Sigma Lab., India. All other chemicals used in this study were of analytical grade. All the measurements were carried out at room temperature.

The stock solution of protein (2 mg/ml) was prepared in 20 mM sodium phosphate buffer of pH7.2 and it was then dialyzed in the same buffer. The concentration of native protein in 20 mM sodium phosphate buffer, pH7.2 was determined from extinction co-efficient of 12.2 A/1 %/1 cm, by UV absorption at 280 nm on a Shimadzu UV-1700 spectrometer.

Effect of Glucose and Fructose on κ-casein

κ-casein was incubated separately in 0.2 M glucose and 0.2 M fructose at physiological temperature. Pure κ casein without sugars was incubated under the same conditions and it was used as control. The final concentration of casein in the incubation mixture at the start of the reaction was 1 mg/ml. All solutions were prepared under sterile condition by adding sodium azide (0.002 %) to avoid contamination (bacterial or fungal) and volume was made up to 3 ml by adding 20 mM sodium phosphate buffer (pH7.2). After proper mixing, samples were incubated at 37 °C for total of fifteen days. After incubation, the samples were extensively dialysed against PBS at 4 °C aliquoted and stored at −20 °C if required. Aliquots were withdrawn for further studies on every third day from the reaction mixtures of control as well as glucose and fructose.

Tryptophan Fluorescence Measurements

The fluorescence spectra were recorded on a Shimadzu RF-5301 spectrofluorophotometer (Tokyo Japan) in a 10 mm path length quartz cell. The excitation wavelength was 295 nm and the emission was recorded in the range of 300–400 nm [15]. The final concentration of protein in sugar samples was 10 μM.

ANS Fluorescence Measurements

ANS binding was measured by fluorescence emission spectra with excitation at 380 nm and emission was recorded from 400 to 600 nm [16]. Typically, ANS concentration was 50 molar excess of protein concentration and protein

concentration was in the vicinity 10 μM for both glucose and fructose samples.

Thioflavin T Assay

Fluorescence spectra were recorded with a Shimadzu RF-5301 spectrofluorophotometer in a 10 mm path length quartz cell. The excitation wavelength was 440 nm and the emission was recorded from 450 to 600 nm. For aggregation studies ThT was added to the samples incubated for different time period, i.e. day 3–15 and then readings were taken and spectra were recorded. Final concentration of protein in the sample was 10 μM for glucose and fructose while the concentration of ThT was15 μM. ThT was prepared in 20 mM sodium phosphate buffer, pH 7.2.

Circular Dichroism Measurements

CD was measured with a JASCO J-815 spectropolarimeter calibrated with ammonium D-10 camphor-sulfonate. Cells of path length 0.1 and 1 cm were used for scanning between 250–190 and 320–250 nm respectively. Each spectrum was the average of 4 scans. Protein concentration for the scans was 10 μM for far- UV and 20 μM for near-UV spectra with sugars.

Fourier Transform Infra-red Spectroscopy

FTIR spectra were recorded with an Interspec-2020 Fourier transform spectrometer in D_2O. Each spectrum was the average of 6 scans. Protein concentration of the samples was typically 40 μM. The scanning wave number was from 1,000–4,000 cm^{-1}.

κ-casein-AGE-associated Fluorescence Analysis

Fluorescence measurements were performed using a Shimadzu RF-5301 spectrofluorophotometer. AGE related autofluorescence of the sample preparations was monitored by exciting at 370 nm and emission wavelength scanning in the range of 400–550 nm. The spectra were corrected with appropriate protein and buffer blanks. Also fluorescence studies at an excitation wavelength of 335 nm and spectra scanning in the wavelength range of 350–500 nm were carried out. For all measurements, the samples contained 10 μM of protein.

Results and Discussion

Tryptophan Fluorescence

The intrinsic fluorophore tryptophan is an excellent parameter to monitor the polarity of the tryptophan environment in the protein and is highly sensitive to the surrounding environment [17]. Due to the presence of a single tryptophan residue at position 76 in κ-casein, it is convenient to determine alterations in the polarity of the environment of tryptophan residue during glycation. Intrinsic fluorescence spectra of κ-casein during glucosylation reaction shown in Fig. 1a indicate changes in protein structure due to reaction with (0.2 M) glucose. The emission spectra of native κ-casein was characterised by emission spectra maxima at 345 nm (curve 1). On incubating the protein during day 3 to 6 (curves 2 and 3) the fluorescence intensity at emission peak 345 nm steadily increases reaching a maximum at day 9 (curve 4). A blue shift of 5 nm in emission maxima (λ_{max} 340 nm) and ~5 % increase in fluorescence intensity was observed on day 9 (curve 4) indicating that the tryptophan residue 76 is getting in a more apolar environment. The increasing trend of fluorescence reverses from the day 12 (curve 5) and reaches near to native, indicating the tryptophan 76 is relocating its native environment, i.e. polar environment. On incubating for 15 days there was further decrease in fluorescence intensity (curve 6) indicating the aggregation of κ-casein. Thus on the basis of tryptophan fluorescence, we can conclude that on day 12, a molten globule state formation is most probably taking place, followed by aggregation on further incubating for 15 days.

Figure 1b shows the intrinsic fluorescence spectra of κ-casein during fructosylation reaction for varying days (0–15). An increase in fluorescence intensity is observed from day 0 to 6 (curves 1–3) with a blue shift of 5 nm on day 6 i.e. λ_{max} at 340 nm, suggesting the apolar residence of tryptophan residue 76 of κ-casein. However, here for (0.2 M) fructose the reversal of the increasing trend of fluorescence is observed at day 9 (curve 4) instead of day 12 as observed in glucose. The close incidence of curve 4 with curve 1 (native) indicates the molten globule state at day 9 with λ_{max} at 340 nm. Incubation for 12 days shows a decrease in fluorescence intensity with emission maxima at 345 nm showing a red shift of 5 nm and implying few aggregate formations in the protein (curve 5). At day 15 a red shift of 10 nm is observed form the native with the emission maxima at 350 nm. The fluorescence is brought down below the native (curve 6) which may be due to the completely unfolded κ-casein molecules which formed the intermolecular aggregates. Thus, we presume at day 15, the presence of detectable aggregates in case of fructose incubated casein.

Figure S1 shows the relative fluorescence of κ-casein as the function of time (day 0–15) with glucose and fructose. Comparison shows that fructose induces the formation of the molten globule state at day 9, faster than glucose (day 12). Moreover, high fluorescence intensity at day 6, along with aggregate formation at day 12 & 15, was observed in the case of fructated casein, which indicates the active

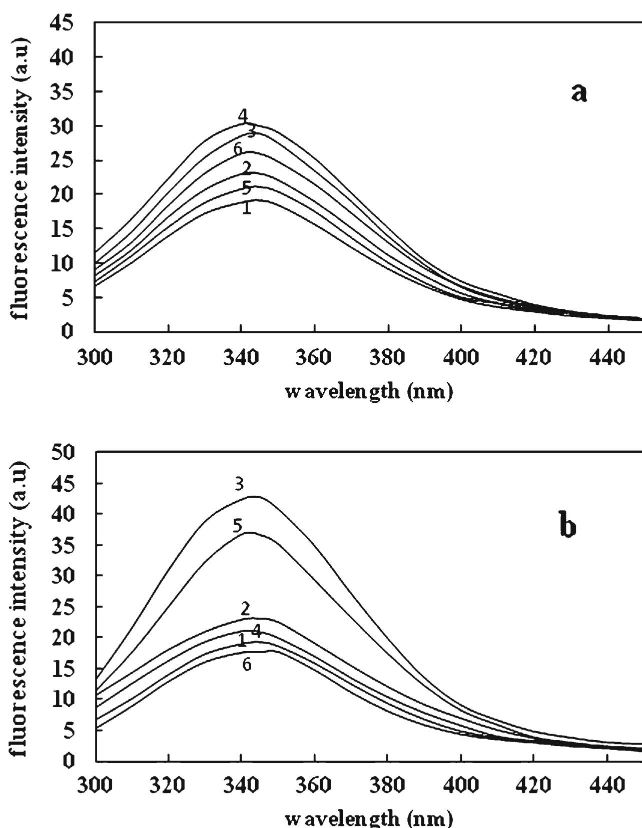

Fig. 1 **a** Tryptophan Fluorescence emission spectra of native κ-casein in 20 mM sodium phosphate buffer pH7.2 (*curve 1*) in presence of 0.2 M glucose incubated for 3 days (*curve 2*), 6 days (*curve 3*), 9 days (*curve 4*), 12 days (*curve 5*) and 15 days (*curve 6*). The protein concentration was 10 μM and the path-length was 1 cm. The fluorescence intensity measurement was carried out at an excitation wavelength of 295 nm and emission was recorded in the range of 300–400 nm. **b** Intrinsic Fluorescence emission spectra of native κ-casein in 20 mM sodium phosphate buffer pH7.2 (*curve 1*) in presence of 0.2 M fructose incubated for 3 days (*curve 2*), 6 days (*curve 3*), 9 days (*curve 4*), 12 days (*curve 5*) and 15 days (*curve 6*). The protein concentration was 10 μM and the path-length was 1 cm. The fluorescence intensity measurement was carried out at an excitation wavelength of 295 nm and emission was recorded in the range of 300–400 nm.

involvement of fructose in inducing structural alteration. Hence, fructose exerts a greater deleterious effect on protein.

ANS Fluorescence

To further explore the sequence of folding and aggregation as shown by first a blue shift and then a red shift of tryptophan emission maximum, the fluorescence measurements were repeated with extrinsic fluorophore ANS incubated with κ-casein. ANS is a hydrophobic dye which is used as an extrinsic fluorophore to monitor the degree of compactness of protein. Figure 2 shows ANS fluorescence for fructated casein. A continuous increase in fluorescence intensity was observed from day 0–6 (curves 1–3) with maximum on day 9 (curve 4). This was followed by a

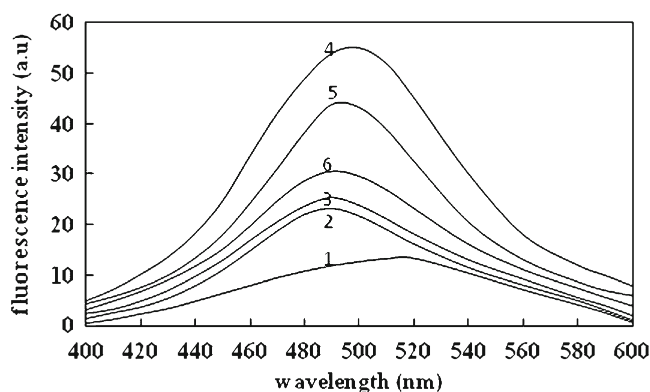

Fig. 2 ANS fluorescence emission spectra of ANS bound to native κ-casein (*curve 1*), in presence of of 0.2 M fructose incubated for 3 days (*curve 2*), 6 days (*curve 3*), 9 days (*curve 4*), 12 days (curve 5) and 15 days (*curve 6*). The excitation wavelength was 380 nm and emission was in the range of 400–600 nm. The protein concentration was 10 μM and the path-length was 1 cm

decrease on day 12 (curve 5) and further less on day 15 (curve 6). Glycation of casein at day 9 apparently opened solvent accessible hydrophobic patches of the protein, making them available for ANS binding and producing a marked increase in its emission intensity and a large blue shift of its emission maxima from 530 nm to 480 nm. Since these are the characteristics of a molten globule, it is characterised as the molten globule state for fructose incubated casein at day 9. With further increase in incubation time for day 12 and 15, this intermediate state gets aggregated leading to the burial of hydrophobic patches causing less ANS fluorescence. These observations thus support the result of tryptophan fluorescence on the time and sugar dependent alterations.

In the case of (0.2 M) glucose reaction with casein slower glycation kinetics was observed with highest fluorescence on day 12 accompanied by a blue shift in spectra maxima from 520 to 480 nm. This is presumably due to the formation of the molten globule state of casein on day 12. When incubated for day 15 there was a decrease in ANS binding resulting in aggregation of the partially folded state of casein (data not shown). The loss in ANS binding can be attributed to the burial of hydrophobic regions among the protein molecules and, therefore, making them less accessible to the ANS fluorophore.

The relative ANS fluorescence as a function of time (day 0–15) on the fructose and glucose induced state of κ-casein is shown in Fig. S2. In comparison of κ-casein incubation with glucose and fructose, the results obtained for the molten globule state via ANS fluorescence are in accordance to that of tryptophan fluorescence. This confirms the formation of molten globule state on day 12 for glucation and day 9 for fructation. The formation of aggregates for fructose begins from day 12 and can be seen to be highest on day 15, whereas for the glucose aggregate, formation is not

seen before day 15; therefore, confirming fructose has a more pronounced effect than glucose on the protein.

To further substantiate the above findings the fructated casein samples was centrifuged. The ANS fluorescence intensity disappears at day 12 and 15 (compared to fructated casein before centrifugation) while for days 3, 6 and 9 remains the same. This confirms the binding of the ANS dye with the aggregates formed on day 12 and 15 which were removed on centrifugation, hence, giving minimum ANS fluorescence intensity in the latter case.

Thioflavin T Fluorescence

The binding of Thioflavin T is considered to be a distinguishing feature of aggregation by proteins. The beta sheet structure and high hydrophobic residues of fructated casein were further assessed by using Th T dye [18]. Fructated casein (Fig. 3) at day 15 (curve 4) showed~100 times higher enhancement than observed for native casein (curve 1). Fructosylated κ-casein at day 12 showed some aggregation enhancement which was ~70 times more (curve 3) than the native. At day 9, the increase in Th T was 30 times more (curve 2) than the native. It seems that the native protein and aggregates can be seen as originating from a common population of partially unfolded and interconverting molecules.

The relative plot of glycated casein with glucose and fructose incubated with Thioflavin T (Fig. S3) is clearly indicative of the fact that early and far more aggregates are formed in case fructosylation than glucosylation. During glucosylation, aggregates are observed at day 15. The aggregate formation during fructosylation begins from day 9 and further increases to a maximum till day 15. Thus fructose causes greater damage than glucose to the protein.

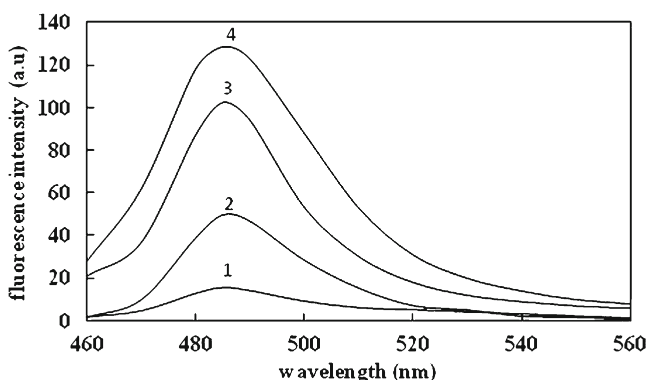

Fig. 3 Thioflavin T spectra of native κ-casein in 20 mM sodium phosphate buffer (*curve1*), incubated with 0.2 M fructose for 9 days (*curve 2*), 12 days (*curve 3*) and 15 days (*curve 4*). The protein concentration was 10 μM and the path-length was 1 cm. The fluorescence intensity measurement was carried out at an excitation wavelength of 440 nm and emission was recorded in the range of 450–600 nm

Circular Dichroism Measurements

Far-UV CD The changes in the secondary structure of glucosylated casein between 190 and 250 nm as a function of time (day 0 to 15) is depicted in Fig. 4a. The spectrum of native κ-casein (curve 1) exhibits minima at 202 nm indicating the presence of a sufficiently random and disordered structure. Incubation of casein for day 3 (curve 2) and day 6 (curve 3) resulted in a decrease in MRE value. Further incubation for 9 days (curve 4) showed a decrease in MRE and appearance of minima at 206 nm, suggesting a red shift of 4 nm. On day 12 (curve 5) CD spectra the same as of the native was observed. Hence, this state can be characterised as a molten globule state. On incubation for 15 days (curve 6) a red shift of 8 nm was observed with minima at 210 nm. Thus from our CD data, we can conclude that at day 12 a molten globule is observed with the characteristic the same as the native and at day 15 indicates structural loss in protein.

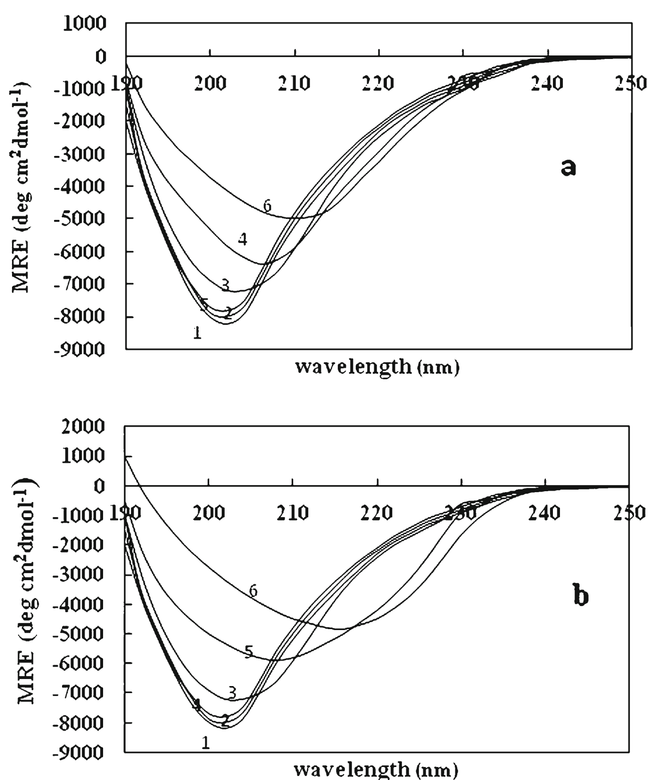

Fig. 4 **a** Far-UV CD spectra of κ-casein in presence of 0.2 M glucose in 20 mM sodium phosphate buffer, pH7.2 at different days of incubation. Curve 1 shows spectra of native casein, κ-casein spectra incubated with glucose for 3 days (*curve 2*), 6 days (*curve 3*), 9 days (*curve 4*), 12 days (*curve 5*) and 15 days (*curve 6*). The protein concentration was 10 μM and the path-length was 0.1 cm. **b** Far-UV CD spectra of κ-casein in presence of 0.2 M fructose in 20 mM sodium phosphate buffer, pH7.2 at different days of incubation. Curve 1 shows spectra of native casein, fructated κ-casein spectra incubated for 3 days (*curve 2*), 6 days (*curve 3*), 9 days (*curve 4*), 12 days (*curve 5*) and 15 days (*curve 6*). The protein concentration was 10 μM and the path-length was 0.1 cm

Fructose at 0.2 M induced conformational changes in the secondary structure of κ-casein as observed by far-UV CD as depicted in Fig. 4b. Here curve 1 for the native state as well as curve 2 at day 3 is having a minima at 202 nm indicating not much change in the secondary structure. Day 6 (curve 3) shows a noticeable decrease in the negative CD signal, suggesting structural alteration. Nevertheless, CD spectra for day 9 (curve 4) revealed MRE value comparable to the native state (curve 1) with a minima at 202 nm, the same as that of the native. Thus, curve 4 (day 9) is more confirmed as a molten globule state. Fructated casein at day 12 (curve 5) and 15 (curve 6) showed a red shift of 6 nm (minima at 208 nm) and of 12 nm (minima at 214 nm) respectively. This suggests the presence of an aggregated state at day 12 and 15, characteristic of the beta sheet structure.

A greater effect of fructose in inducing structural damage to protein is observed by more secondary structure loss on day 12 for fructated κ-casein (Fig. S4) compared to glucated κ-casein on day 9. A similar pattern is observed on day 15. Early induction of the molten globule state (day 9) by the fructose in comparison to glucose (day 12) as confirmed by the far-UV CD studies also substantiates the deleterious effect of fructose.

Near-UV CD Changes in local structure of casein have been reported here by near UV studies. The molar ellipticity of native κ-casein is rather low relative to that of the globular protein. However, the changes observed are not prominent although it does decrease by increasing the incubation time with (0.2 M) fructose or glucose. In the reaction of casein with fructose, the kinetics of glycation was faster and more loss in the tertiary structure of casein was observed as compared to glucose. On incubation with either glucose (Fig. 5a) or fructose (Fig. 5b) for 3 days (curve 2), no significant changes are observed in the local structure of casein which resembles native conformation (curve 1). However, early signs of damage with fructated casein (Fig. 5b) can be seen with loss in the tertiary structure on incubation for 6 days (curve 3) in contrast to glucated casein (Fig. 5a). Further corroboration of this observation comes from early retention of the molten globule state having native like spectral features [19] in case of fructation at day 9 (curve 4) as compared to glucation at day 12 (curve 5). At day 15 (curve 6), there is a maximum loss of the tertiary structure for both glucated and fructated casein.

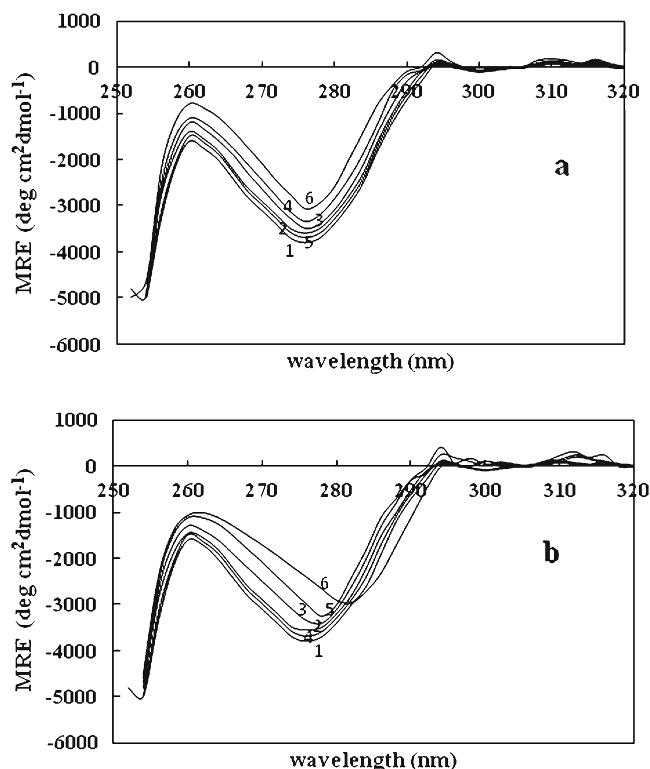

Fig. 5 **a** Near-UV CD spectra of κ-casein in presence of 0.2 M glucose in 20 mM sodium phosphate buffer, pH7.2 at different days of incubation. Curve 1 shows spectra of native casein, glucated κ-casein spectra incubated with for 3 days (*curve 2*), 6 days (*curve 3*), 9 days (*curve 4*), 12 days (*curve 5*) and 15 days (*curve 6*). The protein concentration was 10 μM and the path-length was 1 cm. **b** Near-UV CD spectra of κ-casein in presence of 0.2 M fructose in 20 mM sodium phosphate buffer, pH7.2 at different days of incubation. Curve 1 shows spectra of native casein, fructated κ-casein spectra incubated with fructose for 3 days (*curve 2*), 6 days (*curve 3*), 9 days (*curve 4*), 12 days (*curve 5*) and 15 days (*curve 6*). The protein concentration was 10 μM and the path-length was 1 cm

Fourier Transform Infra-red Spectroscopy

FTIR has been used to probe protein structure, and the amide I band has been used to estimate protein secondary structure content [20]. Infrared spectroscopy predicts β-strands much better than α-helices [21]. Figure 6 depicted wavenumber analysis of κ-casein between 1,580 and 1,700 cm^{-1} revealing a peak corresponding to the region of amide band protein (NHCO). The conformational changes in κ-casein by incubating it with (0.2 M) fructose at the physiological temperature for a period of 15 days were monitored in the amide I region of FTIR spectra. Native casein showed a peak at 1,630 $cm–^{1}$ indicating the presence of intra-molecular β-sheet structure (curve 1). Fructated casein at day 9 shows a peak around 1,630 cm^{-1} (curve 2) with a lower absorbance suggesting that this state is closer to the native β-sheet organisation and can be characterised as a molten globule state. This result is concomitant with the results obtained with the CD data. However, on increasing the incubation time for 15 days there occur a significant drop in absorbance along with the appearance of a broad peak at 1,626 cm^{-1} with a shift of 4 cm^{-1} (curve 3). This is suggestive of the intermolecular aggregated state, i.e. the large amount of β-sheet structures that are formed in

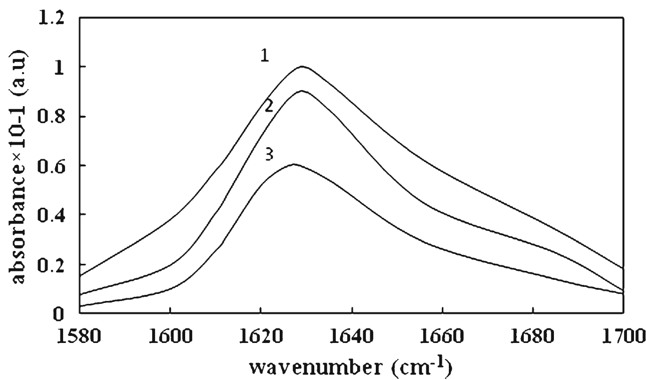

Fig. 6 FTIR spectra of κ-casein in the amide I region in the presence of 0.2 M fructose. Curve 1 shows spectra of native κ-casein in 20 mM sodium phosphate buffer, pH7.2. Curve 2 and 3 depicts κ-casein incubation with 0.2 M fructose for 9 and 15 days respectively. Protein concentration was 40 μM

between the molecules. This type of FTIR spectra was observed in amyloid fibrils confirming the presence of aggregates.

Advanced Glycation End Products Measurements

For highly sensitive detection of AGEs, the fluorescence should be measured at 370 nm [22]. Formation of malondialdehyde (MDA) was assayed by exciting at 370 nm. From Fig. 7 it can be observed that the temporal accumulation of MDA seems to be high in the case of fructose than glucose. The AGE component MDA was formed gradually, starting from day 3 with a significant rise on day 12 and reaching a maximum on day 15 for fructose. Nonetheless, for glucose major MDA, formation can be seen only on day 15.

An excitation maximum of 335 nm for emission at 406 nm for glucose has been reported [23]. This fluorescence accounts

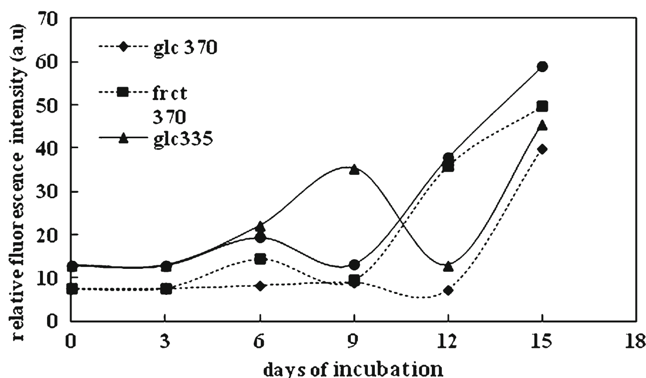

Fig. 7 Time course of two AGEs products (pentosidine (−), excitation 335 nm and malondialdehyde (…), excitation 370 nm) of κ-casein in presence of 0.2 M glucose and 0.2 M fructose. Pentosidine formation when κ-casein is incubated with glucose (*black triangle*) and fructose (*black circle*) for different days (0–15) at 37 °C. Malondialdehyde formation when κ-casein is incubated with glucose (*black diamond*) and fructose (*black square*) for different days (0–15) at 37 °C

for arg-pyrimidine and pentosidine, both well-known AGE structures derived from arginine. In our study (Fig. 7) a small amount of AGEs was detected on day 6 for both glucose and fructose. Accumulation of AGEs elevated further on day 9 in the case of glucated casein. However, formation of these products is brought down to a minimum level on day 9 for fructation and day 12 for glucation. We speculate that such an observation could be due to the formation of molten globule states observed earlier. For fructose, the major amount of AGEs were seen on day 12, which increased to a maximum on day 15, and were even greater than those observed for glucated casein on day 15. Fructose was again most effective in inducing the formation of advanced glycation end products. The tryptophan fluorescence and AGEs formation at 370 nm for various days indicate a similar pattern (fructose being more effective in glycation).

To summarize: we showed the intermediate states of glycation in κ-casein, i.e. molten globule state, aggregates and AGEs as a result of sugar induced modification at the physiological temperature. Comparison of the time scale gives the evidence that the kinetics of structural change induced by fructose is much faster than glucose. Thus, exposure of κ-casein to sugars for a long time period, even at 37 °C, may have a great impact on its structural properties.

Conclusion

The Maillard reaction although has a great potential to generate novel ingredients in edibles, the process is notoriously difficult to control. The processed food when consumed has low digestibility and less nutritive value. This is due to the inactivation of the essential amino acids, inhibition of enzymes and cross-linkage of protein molecules by MR products. Besides, these products have also been associated with increased patho-physiology. It has been shown, [12] that in the bacterial Salmonella typhimurium strain T100, heated sugar-casein systems exhibit mutagenic activities which were remarkably high in ketose-casein mixtures, thus causing considerable chances of cancer. More recently, it was demonstrated that intake of dietary MR products in diabetics promote the formation of pro-inflammatory mediators, leading to tissue injury [24]. Low digestibility and mutagen formation can be attributed to aggregation (causing crosslinking between the protein molecules) and AGEs formation seen at the later stages of glycation. The renal excretion of these dietary ingested AGEs was found to be markedly reduced in diabetics, which could lead to the further advancement of the disease.

Moreover, several canned dairy foods employ the usage of chemical agents (sugar derivatives) as preservatives, which are toxic. Even the readymade milk and other dietary supplements for babies and growing kids available in the market are not left

untouched from the deleterious effects of these preservatives, making them susceptible to damage at the physiological level.

In order to obviate these negative effects of industrial MR, we performed glycation studies at the physiological temperature rather than at higher temperatures. We found that structural changes associated with glycated casein via the Maillard reaction were time and sugar dependant. In the initial phase of advancements of the Maillard reaction, active participation of the free amino ($-NH_2$) group and monosaccharide takes place to form a Schiff's base linkage, leading to an increase in protein fluorescence. However, during the later phase of the reaction, with the gradual decrease in intrinsic fluorescence at 345 nm, the appearance of MDA and pentosidine occur. The fact that the absorption peaks of the two glycation products exhibit strong overlap with the emission peak of tryptophan around 335–350 nm implies the existence of the fluorescence resonance energy transfer (FRET) process. This leads to the quenching of tryptophan fluorescence (at 345 nm) at the expense of the formation of AGE products. At and beyond day 9, significant structural alterations and AGEs formation are observed. Significant changes in secondary structure, molten globule state formation, aggregation, and MDA/ pentosidine accumulation are all clearly noticed for both glucation and fructation for day 9, 12, and 15. Hence, the harmful effects of glycation on κ-casein are indubitable. And since this subset of casein is considered most stable above other subsets, we speculate that other subsets are likely to suffer greater damage. However, we also presume that early stages of glycation induced at physiological temperatures can be safer to be used in the food industry.

Fructose was found to be highly effective making structural alterations in the protein within less time interval. Since glycation via the Maillard reaction has widespread applications in the food industry, taken together, the results of this study presume that if used in safer limits the fructation of casein over glucation can be of better use as an ingredient in food products without the addition of toxic agents. Non enzymatic glycosylation (glycation) of casein, especially fructation, could produce an equivalent functionality with a smaller amount of added protein, helping to reduce the production cost as well as promoting its use as a food ingredient with added value to develop a customer favoured product with a competitive edge in the market place.

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