Unusual spiral structures formed by glycated β-casein in the presence of thioflavin T: amyloid transformation?

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Unusual spiral-like structures in aggregates formed by β-casein glycated in a special way in the presence of thioflavin T are reported. Different glycation agents, temperature, pH, incubation time, and concentrations of protein and modifier were characterized, but only glycated by 200 mM glucose for 48 h at 37 °C without sodium cyanoborohydride β-casein forms spiral structures in the presence of thioflavin T. Thioflavin T affects the size of particles formed by glycated β-casein and also stimulates heat-induced aggregation, indicating that the formation of unusual spiral structures is determined both by the structure of the advanced glycation end products and by the properties of the glycated protein.
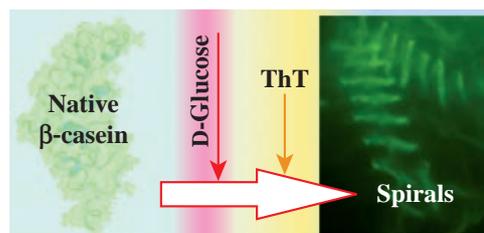
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Protein glycation by various sugars and reactive dicarbonyl compounds is considered to be one of the most important post-translational modifications causing numerous consequences.[1,2] Identification of glycated proteins is widely used to diagnose diseases associated with hyperglycemia, neurodegeneration and aging.[3,4]

The research on food proteins, intensively glycated in the process of cooking at high temperatures in the presence of endogenous or externally added sugars, stands apart.[5] On one hand, the study of food protein glycation is necessary to determine the effect of such a modification on the digestion of proteins in the gastrointestinal tract, the allergenicity of proteins and their effect on gut microbiota.[6,7] On the other hand, food proteins are frequently used as model objects in the study of glycation role in the induction of aggregation and amyloid transformation of proteins.

In a number of works, it was shown that glycation of milk caseins by sugars and aldehydes causes changes in their structure as revealed by traditional methods (circular dichroism, thioflavin T (ThT) fluorescence, *etc.*) and stimulates protein aggregation.[8–10] Along with this, glycation of κ-casein stimulated amyloid transformation and different structural and functional alterations of this protein.[11,12] Unfortunately, information on the relationship between glycation and amyloid transformation of proteins (including caseins) is not well defined and demands careful analysis by both indirect and direct methods to detect amyloid nature of aggregates.[13]

In the present work, we investigated thermal aggregation of glycated bovine milk β-casein (β-CN), paying particular attention to the effect of ThT on the formation of various aggregates, including unusual spiral structures, first discovered here. The observations made herein appear to be important in analyzing the results obtained using the popular fluorescent labeling by ThT, which probably interacts with the advanced glycation end products (AGEs).

The preliminary objective of our work was to study the effect of glycation on the formation of β-CN aggregates when heated. We performed glycation of purified β-CN[14] with 200 mM glucose for 48–72 h at physiological pH and temperature. To identify the possible formation of amyloid structures during thermal denaturation of β-CN we used ThT, since the fluorescence spectra of the latter change upon interaction with β-sheets (see Online Supplementary Materials for all experimental details). As follows from the data in Figure 1, ThT fluorescence intensity added to unmodified β-CN is practically unchanged, while heating at 94 °C results in a 25% increase in fluorescence intensity. However, after addition to glycated β-CN, the ThT fluorescence intensity immediately increases by a factor of 2.5, and is practically unchanged upon further heating. Addition of ThT to sheep prion protein (PrP), which is prone to the formation of amyloid structures upon
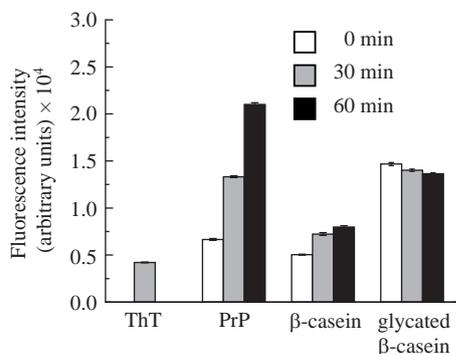
Figure 1 Change in fluorescence intensity of ThT upon binding glycated β-CN. ThT was added to protein samples (at 4 μM final concentration) at a molar ratio of 100:1. PrP – prion protein, unmodified or glycated with glucose β-CN, was incubated at 94 °C during 30 or 60 min.

Figure 3 Transmission electron microscopy of thermoaggregated glycated β-CN after treatment with ThT. 2.5 mM ThT was added to glycated β-CN samples (0.06 mM in 20 mM Tris-HCl buffer, pH 7.5) after thermal denaturation at 94 °C for 60 min and incubated for 10 min. Samples were spotted onto a grid and stained with uranyl acetate.

heating, results in gradual increase of ThT fluorescence intensity, reaching a three-fold increase in 60 min and revealing the initial stages of amyloidization.[15] These observations indicate that changes in ThT fluorescence parameters are caused by initially present or rapidly formed structures in the case of glycated β-CN compared to PrP. Thus, the presented data shows that ThT interacts with glycated forms of β-CN, but this interaction may be unrelated to the formation of its complexes with β-sheets, since the increase in ThT fluorescence intensity is immediate and is not sustained throughout incubation as in the case of amyloid PrP.

According to light microscopy results, there are practically no visible aggregates in unmodified and glycated β-CN. After heating for 60 min at 94 °C amorphous aggregates appear in both unmodified and glycated β-CN samples, and their number is much higher in the case of glycated β-CN. The addition of ThT to unmodified β-CN does not significantly affect its aggregation. However, glycated β-CN forms large aggregates after the addition of ThT even before heating. The structure of aggregates formed in the presence of ThT was studied using fluorescence microscopy (Figure 2). Unmodified β-CN preparations contain a small amount of amorphous aggregates that exhibit weak fluorescence after ThT staining [Figure 2(*a*),(*b*)]. A completely different situation is observed for β-CN glycated with glucose. After addition of ThT and incubation for 10 min, glycated β-CN forms large aggregates with high fluorescence intensity, consisting of spiral structures [Figure 2(*c*)]. ThT fluorescence intensity upon interaction with
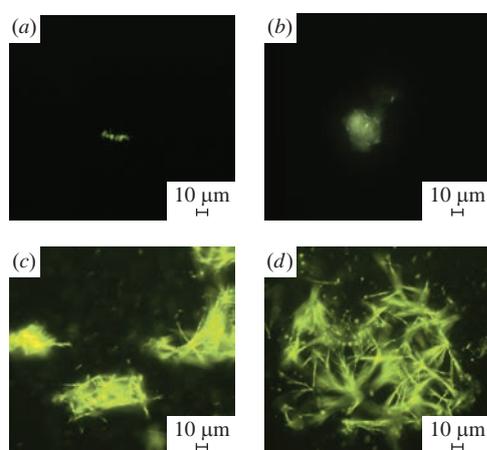
heliciform structures increases after thermal aggregation, and the aggregates themselves become more compact [Figure 2(*d*)].

The structure of spiral aggregates shown in Figure 2(*d*) was studied in more detail by transmission electron microscopy. As follows from images in Figure 3, samples of glycated β-CN after the addition of ThT contained spiral structures. Spiral sections in the middle of β-CN aggregates are indicated by arrows [Figure 3(*a*)]. In Figure 3(*b*), the spiral portion is shown at a higher magnification.

Further confirmation of the data on the effect exerted by ThT on glycated with glucose β-CN aggregation was obtained using turbidimetry and dynamic light scattering methods (see Online Supplementary Materials).

It was necessary to determine the composition of the detected spiral aggregates, as well as to establish the influence of β-CN glycation parameters on the formation of these unusual structures upon ThT addition.

First of all, it was proven that the spiral aggregates really consist of protein molecules, namely, of the β-CN molecules. Incubation of the spiral structures with ninhydrin led to the appearance of violet color, indicating the presence of protein. Immunochemical staining of spiral aggregates with antibodies specific to β-CN,[16] followed by fluorescence microscopy, also confirmed the presence of this particular protein. It should also be noted that spiral structures were not found in ThT solutions, as well as in preparations of α-lactalbumin which was glycated in the same way as β-CN.

It is known that the modification of proteins by glucose results in the formation of AGEs that have a complex aromatic structure. We hypothesized that it is the AGEs contained in β-CN after prolonged treatment with glucose that could interact with ThT, altering its fluorescence intensity and stimulating the formation of spiral structures. To verify this hypothesis, we obtained preparations of β-CN glycated in various ways and compared their ability to form spiral aggregates in the presence of ThT. β-CN was incubated in the presence of 200 mM glucose at 37 °C for 48 and 72 h with or without sodium cyanoborohydride (to reduce the Schiff bases). In addition, β-CN was incubated in the presence of 200 mM methylglyoxal at 37 °C for 12 and 24 h. The addition of the reducing agent, sodium cyanoborohydride, was intended to decrease the formation of AGEs during glycation. The modification by methylglyoxal was aimed to decrease the content of fluorescing aromatic AGEs and promote the formation of simple products like carboxyethyllysines. Table 1 summarizes data on the content of the early glycation products, free amino groups, and AGEs in the preparations of β-CN treated under different conditions. As follows from these data, methylglyoxal modifies β-CN more efficiently, and the addition of sodium cyanoborohydride leads to an increase in the number of free amino groups and a decrease in the content of AGEs. It was found that the fluorescence intensity of ThT increased when interacting with all the studied preparations of glycated β-CN, although to a different degree. Glycation also stimulated the thermal aggregation of β-CN. However, comparison of different preparations of glycated β-CN using fluorescence microscopy showed that unusual spiral aggregates are formed only when

Figure 2 Fluorescence microscopy of ThT-treated β-CN before (*a*, *c*) and after (*b*, *d*) heating at 94 °C for 60 min. (*a*), (*b*) β-CN without modifications, (*c*), (*d*) β-CN after 48 h of incubation with 200 mM glucose. 2.5 mM ThT was added to β-CN samples (0.06 mM in 20 mM Tris-HCl buffer, pH 7.5), after thermal denaturation at 94 °C for 60 min in case of (*b*), (*d*) and incubated for 10 min. Images were captured with exposure time 100 ms.
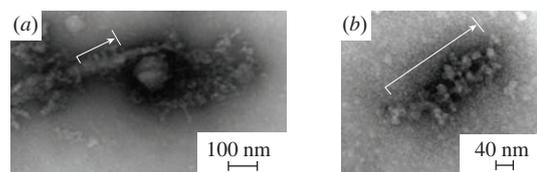
Table 1 Characterization of β-CN modified with glucose and methylglyoxal under different conditions.[a]

Conditions of the β-CN modification	Incubation time / h	Contents of early glycation products (by fructosamine assay)		Free amino groups in modified β-CN (%)	Detection of AGEs using fluorescence spectroscopy (arbitrary units)
		Number of modified amino groups per mole of β-CN	%		
Before modification		0	0	100	4936 ± 109
200 mM Glc	48	6.1 ± 0.4	36	–	18765 ± 456
200 mM Glc	72	7.6 ± 0.5	45	54	32812 ± 690
200 mM Glc + + 35 mM NaBH$_3$(CN)	72	6.6 ± 0.5	38	71	26574 ± 622
200 mM MGO	12	5.2 ± 0.5	31	62	8624 ± 522
200 mM MGO	24	12.8 ± 0.5	75	21	15058 ± 388
200 mM MGO + + 35 mM NaBH$_3$(CN)	24	10.2 ± 0.5	60	36	12387 ± 421

[a] Glc is glucose, MGO is methylglyoxal.

ThT is added to β-CN glycated by glucose without the addition of the reducing agent. The addition of ThT to β-CN modified with methylglyoxal and also with glucose in the presence of a sodium cyanoborohydride results in the formation of amorphous fluorescent aggregates. It can be assumed that the complex aromatic AGEs that are formed when β-CN is modified by glucose in the absence of the reducing agent interact with ThT, leading to the formation of spiral structures.

The presented data indicate that ThT interacts with glycated forms of β-CN, but no changes in β-sheets content were revealed using other methods. This interaction of glycated β-CN with ThT leads to an increase in the intensity of its fluorescence, but to a lesser extent than in the case of binding to β-sheet structures of amyloid proteins. ThT also affects the size of the particles formed by glycated β-CN and stimulates heat-induced aggregation of such a modified protein. These effects were found on the preparations of β-CN, modified by different techniques – prolonged incubation with glucose or a relatively rapid modification by methylglyoxal. Glycation in the presence of a reducing agent sodium cyanoborohydride had no significant effect on the ability of ThT to interact with β-CN and alter its properties. However, β-CN, glycated for 48 h by glucose without the addition of a reducing agent, upon addition of ThT forms unusual spiral-shaped structures that can be observed with fluorescence or transmission electron microscopy. The presence of protein in these structures, revealed with ninhydrin reaction, as well as immunostaining of spiral aggregates with antibodies to β-CN, indicates their protein nature. When β-CN is modified with methylglyoxal or under different conditions for modification by glucose, aggregation of β-CN or glycated in the same way lactalbumin occurs, but no spiral structures are formed.

The results appear to be important for several reasons. First of all, ThT binding to glycated proteins (β-CN and α-lactalbumin) that causes a change in the fluorescence of the dye indicates the possibility of its use for identification of glycation products in proteins. The possibility of increasing the fluorescence of ThT in its interaction with glycated proteins should be taken into account while using this reaction to detect amyloid structures to prevent false-positive reactions. Second, ThT can be applied to regulate the processes of aggregation of glycated proteins, including stimulation of the formation of condensed spiral structures instead of amorphous aggregates. And last but not least, the interaction of ThT with β-CN containing certain AGEs leads to the formation of unusual spiral structures. Such nanoscale structures containing food proteins can be used to deliver ThT as anti-amyloid agent.

For further work we plan, first of all, to study the effect of ThT on the formation of complexes between glycated proteins (glycated caseins at the first stage) and amyloidogenic proteins (prion protein). Based on our preliminary data, we believe that ThT will significantly alter the structure of aggregates of these two proteins, stimulating the formation of unusual forms. The study of these processes is particularly important because ThT is suggested as a compound that prevents the amyloid transformation of proteins and even causing their destruction. The effect of ThT on the aggregation of glycated proteins and their possible participation in the formation of amyloid aggregates should be considered when using ThT and its analogs as a prophylactic and medication.

Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi: 10.1016/j.mencom.2021.01.022.

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