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Aggregation behavior of casein is correlated with the type of glycation-inducing agent

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ABSTRACT

It has been discovered that protein glycation is involved in several human degenerative diseases and/or disorders, such as diabetes and Alzheimer's disease. Evidence suggests that protein glycation results in the formation of fructosamine and advanced glycation end-products (AGEs), accompanied by the alteration of protein conformation. To investigate how casein is influenced by glycation induced by ribose or methylglyoxal, structural characterization was carried out using thioflavin T (ThT) binding assay, far-UV circular dichroism, intrinsic fluorescence spectroscopy, light scattering, turbidity measurement, and transmission electron microscopy. AGE-related fluorescence measurement and nitro blue tetrazolium assay were used to examine the information regarding the pathway and relevant products associated with glycation. Degree of modification was characterized by determining the contents of lysine and arginine residues. We found that treatment of casein with glycation-inducing agents/glycation modifiers led to an obvious structural unfolding and enhanced solvent-exposed hydrophobic regions. In addition, we found that the concentration profile and production rate of fructosamine and/or AGEs were dependent on the type of glycation-inducing agent used. Moreover, our results showed that amyloid/amyloid-like aggregated species, with relative higher β-sheet secondary structure content and ThT fluorescence-positive characteristics, were found only when casein was treated with methylglyoxal. Finally, modification with methylglyoxal resulted in a higher percentage of reacted lysine or arginine residue in the casein sample. We believe the results of this work could aid in understanding the process of protein glycation.

1. Introduction

Protein glycation occurs anytime proteins come into contact with sugars. Any given protein can react with any form of reducing sugar; the main difference is that different proteins and reducing sugars have different reactivities, resulting in distinct process reaction rates. Age and sugar concentration can induce an increased degree of glycation within the body [1]. The glycation of proteins normally takes place slowly inside the human body; however, in diabetic patients, the process occurs at a much higher rate, due to the higher blood sugar levels in their systems [2]. Thus, it can be deduced that glycation might be the cause of diabetes-related complications.

Non-enzymatic glycation, also known as the Maillard reaction, occurs when sugar molecules are bound with proteins without the presence of controlling enzymes [3]. The first step of the Maillard reaction involves a nucleophilic attack on the carbonyl group of the reducing sugars by the nitrogen on the protein, leading to the formation of a Schiff base. The Schiff base is rearranged into fructosamine, also known as the Amadori product, which is then oxidized into α-dicarbonyl compounds known as early glycation products. Further oxidation of the early glycation products produces advanced glycation products (AGEs) [4].

Studies have also indicated that glycation can be responsible for the alteration of protein structures, along with protein misfolding and the formation of amyloid fibrils, a type of ordered protein aggregates [3]. These changes in protein structure can result in the failure of several biological functions and cause degenerative disorders such as Alzheimer's disease and cataract [5]. Using diabetic animals as a model system, Amadori product antibodies and aminoguanidine (an inhibitor of advanced glycation) have been shown to diminish, to a degree, the pathological effects of diabetes [6].

Evidence indicates that glycated proteins are present in amyloid deposits in a wide range of amyloid-associated diseases, including β-amyloid deposits and Tau aggregates in Alzheimer's disease (AD) [7,8], Lewy inclusion bodies of α-synuclein in Parkinson's disease

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[9], and transthyretin amyloid deposits in familial amyloidotic polyneuropathy (FAP) [10]. In these amyloid pathologies, the presence of AGE and/or the formation of β-sheet-rich fibrillar aggregates are common features/characteristics. In addition, glycation has been found to accelerate the formation of cross-β structure of β-amyloid (1–42) peptide and islet amyloid polypeptide (IAPP) [11,12]. While the detailed mechanism(s) of how glycation is involved in the formation of amyloid deposits remain(s) elusive, the lines of evidence mentioned above apparently suggest a possible role for glycation in the pathogenesis of amyloidosis.

Ribose is a pentose monosaccharide that is naturally present in all living cells. In the human body, ribose can also be converted from glucose through the pentose phosphate pathway; it has ample opportunity to react with proteins and produce glycated products, as it is present both intra- and extracellularly [4]. Compared to glucose, ribose has a more active effect on the glycation of proteins accompanied by the formation of AGEs. In addition to ribose, methylglyoxal, one of the reactive dicarbonyl compounds, is also capable of triggering glycation. Methylglyoxal has been shown to selectively modify arginine and lysine residues, with arginine being the primary target [5]. Reports indicated that methylglyoxal reacts reversibly with arginine, lysine, and cysteine residues in proteins; however, it can further react with lysine and arginine irreversibly to form glycosylamine protein crosslinks and imidazolone derivatives, respectively [13].

Casein, the most common milk protein, makes up approximately 70–80% of cow's milk (~26 g/L). Due to its nutritional value and sensitivity to acid, casein has been manufactured into numerous food products, such as cheese and yogurt. Casein is a combination of phosphoproteins and has an average molecular weight of 24 kDa. With its flexibility and structurally unfolded nature, casein does not have clearly defined secondary and tertiary structures; thus, it is known as an intrinsically disordered protein [14]. However, evidence has indicated that the casein subunits α_{S2}- and κ-caseins, display amyloid fibril-forming propensities under physiological conditions [15,16].

In the current work, using casein as a model protein, turbidity measurement, intrinsic fluorescence spectroscopy, far-UV circular dichroism (CD) spectroscopy, thioflavin T (ThT) fluorescence spectroscopy, and transmission electron microscopy (TEM) were used to examine how protein structure, along with its aggregation behavior, was affected by glycation induced by ribose or methylglyoxal. We found that the glycation/modification of casein by two different glycation-inducing agents led to different outcomes in terms of degree of aggregation, extent of structural unfolding, and changes in secondary structure. In addition, when casein was treated with ribose, the amount of AGEs present and the concentration of fructosamine formed were found to be considerably higher than those of the methylglyoxal-treated casein sample. Moreover, the ThT binding assay, TEM, and far-UV CD spectroscopy showed that amyloid/amyloid-like fibrillar species were detected in the samples containing methylglyoxal-treated casein, but not in the untreated casein and ribose-glycated casein samples. Finally, our data indicated that higher percentages of reacted amino groups (including lysine and arginine residues) were present in the samples of methylglyoxal-treated casein, suggesting greater methylglyoxal reactivity toward proteins as compared with ribose. We believe the results of this work could contribute to a better understanding of the protein glycation process.

2. Materials and methods

2.1. Materials

Hydrochloric acid and sodium chloride were purchased from Nacalai Tesque, Inc. (Japan). Casein, ribose, methylglyoxal (MG), and other chemicals, unless otherwise specified, were purchased from Sigma (USA).

2.2. Preparation of casein sample solutions

All casein solutions (~5 mg/mL), in the presence and absence of various concentrations of glycation-inducing agents (reducing sugar: ribose; reactive dicarbonylic compound: methylglyoxal), were prepared immediately prior to each experiment by dissolving the casein powders in Tris–HCl buffer solution (20 mM, pH 7.4) with 1.54 mM NaN_3. At designated time points, an appropriate amount (~800 μL) of casein solution was withdrawn and then dialyzed against 400 mL Tris–HCl buffer solution for 8 h (buffer was changed every 4 h) in a dialysis bag (Sanko Junyaku Co., Ltd., Japan) to remove the small molecules from the casein sample solution. The resultant dialyzed casein sample solutions, at appropriate concentrations, were used for the following measurements. The protein concentrations of the resultant dialyzed casein sample solutions (untreated and treated with either glycation-inducing agent) were determined using a bicinchoninic acid (BCA) assay [17], with casein as the standard.

2.3. Thioflavin T (ThT) fluorescence spectroscopy

A stock solution of ThT at a concentration of ~500 μM was prepared in 95% (v/v) ethanol protected from light prior to use, and the concentration was determined spectrophotometrically using a molar extinction coefficient of 416 nm of 26,600/M/cm [18]. Tris–HCl buffer (20 mM, pH 7.4) was used to dissolve the ThT stock solution to obtain a ThT working solution at a concentration of 10 μM. The dialyzed casein sample solutions (3 mg/mL, 40 μL), untreated and treated with either glycation-inducing agent and taken out at different incubation times, were mixed thoroughly with the ThT working solution (960 μL) prior to measuring the ThT fluorescence emission intensities at 485 nm. The ThT fluorescence measurements were taken for 60 s by exciting the resultant mixtures at 440 nm with a Cary Eclipse fluorescence spectrophotometer (Varian, USA).

2.4. Far-UV circular dichroism (CD) spectroscopy

CD spectra of the dialyzed casein samples were recorded after diluting 10-fold with deionized water over a wavelength range of 190–260 nm, using a J-815 spectrometer (JASCO, Japan) with a 0.2-cm path length sample cell. All CD measurements were collected at room temperature using a bandwidth of 2.0 nm, a step interval of 0.1 nm, and a scanning speed of 50 nm/min. Each CD spectrum was the average of three scans. Control buffer scans were run in duplicate, averaged, and then subtracted from the sample spectra. The secondary structure contents (α-helix, β-sheet, turn, and random coil) of the samples were estimated using the CDSSTR algorithm with reference set 7 available from the DICHROWEB website [19].

2.5. Right-angle light scattering

Scattered light intensities (at a fixed angle of 90°) were detected in the 3 mg/mL dialyzed casein samples (untreated and treated with either glycation-inducing agent) with an F-2500 fluorescence spectrophotometer (Hitachi, Japan), at equal excitation and emission wavelengths of 450 nm.

2.6. Turbidity measurement

The turbidity measurements of the 3 mg/mL dialyzed casein samples (untreated and treated with either glycation-inducing