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Analytical Methods

Monitoring protein glycation by electrospray ionization (ESI) quadrupole time-of-flight (Q-TOF) mass spectrometer

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ABSTRACT

In this study electrospray ionization quadrupole time-of-flight (ESI-Q-TOF) mass spectrometry was used to investigate protein glycation. The glycated species of cytochrome C, lysozyme, and β-casein formed during glycation with d-glucose were identified and monitored in binary systems heated at 70 °C under dry and aqueous conditions. Cytochrome C had multiple charges in non-glycated state, primarily changing from +13 to +17 positive charges, whereas β-casein had charge states up to +30. Upon heating with glucose at 70 °C in aqueous state, attachment of one glucose molecule onto proteins was observed in each charge state. However, heating in dry state caused much more glucose attachment, leading to the formation of multiple glycoforms of proteins. By using ESI-QTOF-MS technique, formation of glycated cytochrome C containing up to 12 glucose moieties were observed, while glycated species containing 6 and 8 glucose moieties were observed for lysozyme and β-casein, respectively in various heating conditions.

1. Introduction

Glycation is the addition of a sugar moiety into a protein molecule, and occurs during Maillard reaction, which is a series of complex reactions including condensation, elimination, and degradation mechanisms. In the early stage of the reaction, sugar molecule reacts with the amino group of protein to give the unstable Schiff base, which undergoes spontaneously a rearrangement to form an Amadori or a Heyn's product. In the intermediate stage these products degrade to reactive carbonyl compounds via dehydration and oxidation. These products are much more reactive than the precursor sugar molecules towards the amino groups, and reaction continues further. In the later stages of the reaction, irreversible compounds are formed through oxidation, dehydration, and cyclization reactions. The reaction mechanism was first described by Hodge (1953) and then more description of the reaction route was established by other researchers (Thornalley et al., 2003; Yaylayan, 1997). The so-called Advanced Glycation Endproducts (AGEs) lead to several consequences within the body; they may take part in chronic and degenerative disorders such as diabetes and renal failure (Sebekova & Somoza, 2007), atherosclerosis (Wang et al., 2012), Alzheimer's and Parkinson's diseases (Li, Liu, Sun, Lu, & Zhang, 2012). It is known that the formation of AGEs increases with normal aging and age-dependent AGEs generally accumulate in long-lived proteins such as lens proteins and collagen (Odjakova, Popova, Al, & Mironova, 2012). AGEs are also formed in higher rates in case of diabetes, where serum glucose levels, glyoxal, methylglyoxal, and 3-deoxyglucosone concentrations are increased, and in uremia where α-oxoaldehydes concentrations are increased (Thornalley et al., 2003). Besides reactions taking place in biological systems, glycation also occurs during certain food processing conditions such as baking, roasting, frying, drying, and pasteurization/sterilization. This type of glycation reaction leads to impairment of nutritional quality of food and generation of undesired compounds called process contaminants; such as acrylamide and hydroxymethyl furfural. Dietary intake of exogenous AGEs may cause their accumulation in blood stream and in tissue proteins (Baynes, 2001), then undergoing further reactions in the body (Vlassara, 2005). Therefore, investigation and screening of protein glycation has been of interest of both medical and food scientists.

Potential glycation sites of a protein molecule can be listed as ε-amino group of lysine, guanidine group of arginine, thiol group of cysteine or any *N*-terminal amino group of amino acids. Besides the environmental conditions such as temperature, water activity, and pH, the characteristics of the reactants themselves are important in terms of the progression of glycation. Yaylayan (1997)

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defined a reactant pool, comprising of amino acids, sugars, and the Amadori or Heyn's products, and their degradation products. The nature and their relative ratios in the pool would determine the pathway of Maillard reaction under specific conditions (Yaylayan, 1997). Consequently, the monitoring of glycation reaction is a challenging issue due to the complexity of the reaction.

Glycation products have been monitoring in medical field and in food science investigations by various analytical techniques. Absorbance measurements (Davies et al., 1998; Labuza & Saltmarch, 1982), fluorescence measurements (Morales, 1996; Morales & van Boekel, 1997; Suarez, Etlinger, Maturana, & Weitman, 1995), and fluorescamine assay (Yaylayan, Huyghues-Despointes, & Polydorides, 1992; Yeboah, Alli, & Yaylayan, 1999) can be listed as the methods giving only cumulative information about the glycation. On the other hand, in some other techniques, glycation markers are quantitatively analyzed, such in the case of furosine (the compound formed from acidic hydrolysis of Amadori product, fructoselysine) and CML analysis (Drusch, Faist, & Erbersdobler, 1999; Krause, Knoll, & Henle, 2003; Kubow, Yaylayan, & Mandeville, 1993; Moreaux & Birlouez-Aragon, 1997). However, although the latter techniques give more precise results, these techniques require acidic hydrolysis application, which is time and chemical consuming. Mass spectrometric techniques have also been using for the quantitative and qualitative analysis of glycation successfully both in food and biological systems, especially MALDI and ESI mass spectrometry (Humeny, Kislinger, Becker, & Pischetsrieder, 2002; Kislinger, Humeny, Seeber, Becker, & Pischetsrieder, 2002; Lapolla, Fedele, Seraglia, & Traldi, 2006; Thornalley et al., 2003; Yeboah, Alli, Yaylayan, Konishi, & Stefanowicz, 2000).

This study aims to develop an electrospray ionization (ESI) quadrupole time-of-flight (Q-TOF) mass spectrometer based analytical approach for evaluating protein glycation. The glycated species of lysozyme, cytochrome C, and β-casein formed during their glycation with d-glucose were identified and monitored in binary systems heated at 70 °C for different times under dry and aqueous conditions.

2. Materials and methods

2.1. Materials

Lysozyme from chicken egg white (UniProtKB-P00698 [LYC_CHICK]) (62970) was purchased from Fluka, Cytochrome C (UniProtKB-P00004 [CYC_HORSE]) from horse heart muscle was purchased from Acros Organics (Geel, Belgium). Bovine β-casein (UniProtKB-P02666 [CASB_BOVIN]), acetonitrile, methanol and formic acid were supplied from Sigma-Aldrich Co. (Germany) and used without purification. Water used in experiments was purified by using Puris-Expe Ultrapure Water System (Mirae St. Co. Ltd, Korea).

2.2. Preparation of model systems

In order to investigate the glycation trend of proteins, model systems of lysozyme, cytochrome C, and β-casein were prepared. Lysozyme C [19–147] is a 129 amino acid long chained protein, having an average molecular mass of 14305.1 Da, whereas cytochrome C has a chain length of 104 amino acids and having 12360.2 Da average molecular mass. Lysozyme C has 8 mol of lysine, 10 mol of arginine, 3 mol of glutamine, 13 mol of asparagine residues, while cytochrome C has 19 mol of lysine, 2 mol of arginine, 3 mol of glutamine, and 5 mol of asparagine residues per protein molecule. They have almost 30 amine groups per protein molecule. Therefore 1 μmol of these proteins were mixed with 30 μmol of glucose in 1 mL of water. β-casein has a molecular mass of almost 24 kDa and 224 amino acid residues. It has 12 mol of lysine, 4 mol of arginine, 20 mol of glutamine, and 5 mol of asparagine residues per protein molecule. Therefore, 1 μmol of β-casein was mixed with 41 μmol of glucose in 1 mL of water. The mixtures were glycated in aqueous or in dry state after freeze-drying. The glycation reactions were performed in tightly closed test tubes containing the mixtures. The tubes were placed in a water bath and heated at 70 °C for different times up to 24 h for lysozyme and cytochrome C, and up to 6 h for β-casein.

For comparison, 1 μmol of lysozyme, cytochrome C and β-casein were also prepared without glucose and heated at 70 °C for different times as control.

2.3. Electrospray ionization (ESI) mass spectrometry

Mass spectrometric analysis of model proteins were performed, with an Agilent 6530 electrospray (ESI, Dual JetStream) quadrupole time-of-flight (QTOF) mass spectrometer (Santa Clara, CA). Glycated and non-glycated protein samples were dissolved and/or diluted in the mixture of acetonitrile:water:formic acid (1:1:0.001 v/v/v) mixture ($\approx 10^{-5}$ M) and they were directly introduced into the instrument at a rate of 10 μL/min using a syringe pump. Nitrogen was used as the nebulizer, desolvation and cone gas. The Jet Stream conditions applied for positive ionization were: capillary voltage 4500 V, fragmentor voltage 100 V, gas temperature 300 °C, drying gas flow 8 L/min, nebulizer pressure 45 psi, sheath gas temperature 350 °C, sheath gas flow 10 L/min. Samples were usually detected in the low mass range (m/z 1700) window and high resolution mode of the instrument. However, the other instrument modes such as extended dynamic range were also used when signal intensity is not sufficient to follow some species. Instrument was tuned and externally calibrated (10 point) using Agilent ESI mass spectrometer tuning mixture.

3. Results and discussion

Fig. 1 shows glycation profile of cytochrome C heated in aqueous state. It can be seen that cytochrome C has multiple charges in non-glycated state, primarily changing from +13 to +17 positive charges (shown in the range of m/z 720–980) (Fig. 1A). Observed m/z values of glycated and non-glycated cytochrome C signals are listed in Table 1. It can also be seen that the most abundant charge states are +15 and +16, which represents the most stable charge states of cytochrome C at applied experimental conditions. Upon heating of cytochrome C with glucose at 70 °C in aqueous state, binding of one glucose molecule to one Cytochrome C was observed in each charge states (Fig. 1B–E). The mass difference between adjacent cytochrome C and glycated cytochrome C signals was 162 Da, which is equivalent to the mass of a water loss after glycation ([M + Glucose-H_2O]). Upon glycation, the trend in the predominant masses did not change except in the sample heated for 24 h. In the sample glycated during 24 h in aqueous state, signal response of +17 charged protein was higher (Table 1), which means that glycation changed the ionization behavior of cytochrome C.

When the signals of glycated samples are compared, it can be seen that signal intensities associated with single glucose adduct increased with glycation time. This means that progression of glycation reaction could be easily determined by monitoring the glycated protein signals at specified charge states, such as; $[M + Glucose\text{-}H_2O + 13H]^{13+}$, $[M + Glucose\text{-}H_2O + 14H]^{14+}$, $[M + Glucose\text{-}H_2O + 15H]^{15+}$, $[M + Glucose\text{-}H_2O + 16H]^{16+}$, and $[M + Glucose\text{-}H_2O + 17H]^{17+}$ signals having nominal masses 964, 895, 835, 783, 737 Da respectively for cytochrome C heated with glucose in solution under the stated conditions.