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Characterisation of bovine serum albumin–fucoidan conjugates prepared via the Maillard reaction

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ABSTRACT

Bovine serum albumin (BSA)–fucoidan conjugates were prepared by the Maillard reaction (60 °C and 79% relative humidity for 96 h), and were then identified by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and size-exclusion chromatography (SEC). Molecular characteristics of the BSA–fucoidan conjugates were investigated, using atomic force microscopy (AFM), dynamic light scattering (DLS), fluorescence spectroscopy, and circular dichroism spectroscopy. SDS–PAGE patterns provided evidence for the covalent bonding between BSA and fucoidan. SEC profiles showed that about 1.5–2.0 mol of fucoidan were covalently linked to 1 mol of BSA, resulting in high-molecular-weight compositions (conjugates). AFM images and DLS results indicated that most particles in the conjugates were nano-structured and more spherical than those of a regular BSA–fucoidan mixture. The fluorescence intensity and maximum emission wavelength of the conjugates together revealed that the BSA molecules had converted from an ordered conformation into a partially folded molten globule state.

1. Introduction

The Maillard reaction involves the non-enzymatic binding of glycans to a wide variety of proteins, to form glycoconjugates (Varki et al., 2009, chap. 1). In this reaction, an available amino group (mainly the ε-amino group of a lysine residue in a protein) interacts with the carbonyl group of a reducing carbohydrate to form a Schiff base. With the loss of water, the labile Schiff base rearranges to form an Amadori product (Horvat & Jakas, 2004). This protein modification procedure is considered to be potentially useful in the food industry, because it improves the physicochemical and functional properties of proteins without the need for the addition of extra chemical reagents or enzymes (Li et al., 2009).

Bovine serum albumin (BSA) is a model protein that is used widely in food systems. The globular-shaped BSA molecule is composed of a single polypeptide chain with a molecular mass of 66.5 kDa. Globular proteins can exist in different conformations (namely, ordered, molten globular, pre-molten globular, and unfolded), with each form having structural differences and increasing amounts of disorder (Uversky & Ptitsyn, 1994). Molten globules have a nearly 100% native secondary structure and a 15% and 50% increase in size and volume, respectively, compared with the native form; however, these changes are much less than the completely unfolded form (Uversky, 2002). Shin and Hirose (1995) have shown that the disulphide-reduced form of the domain-I-truncated fragment adopts a molten globule-like state. Each molecule of BSA contains 60 lysine residues, all of which have free amino groups that are accessible for the Maillard reaction (Huang, Kim, & Dass, 2004).

Recently, increasing attention has been focussed on improving the functional properties of BSA by conjugating it with several carbohydrates via the Maillard reaction. Of the conjugates of BSA with D-glucose (Glc), D-allose (All), and 6-*O*-octanoyl-D-glucose (GlcC8), the BSA–GlcC8 conjugate showed greater improvement in interfacial and emulsifying activities than did BSA–Glc and BSA–All, but no improvement in emulsion stability (Rangsansarid, Cheetangdee, Kinoshita, & Fukuda, 2008). Berthold, Schubert, Brandes, Kroh, and Miller (2007) also reported that BSA modified with glucose and maltose, or by acetylation, had enhanced surface activity as compared with native BSA. For the stabilization of emulsions, BSA–galactomannan conjugates showed a higher emulsion stability index than did native BSA or a regular mixture of BSA and galactomannan (Kim, Choi, Shin, & Moon, 2003). Some studies have concentrated on the structural characterisation of BSA conjugates (Jung, Choi, Kim, & Moon, 2006; Shi, Sun, Yu, & Zhao, 2010). Other reports have focussed on the biological activities of Maillard reaction products of BSA. For example, neoglycoconjugates, containing chitin oligosaccharides, could be used to search for prophylactic agents for bacterial infections (Ledesma-Osuna,

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Ramos-Clamont, Guzman-Partida, & Vazquez-Moreno, 2010). Despite numerous observations on the properties of Maillard reaction products of BSA, there is little information available on BSA–fucoidan conjugates.

Fucoidans are sulphated fucans that are extracted from brown seaweed. Fucoidans extracted from *Undaria pinnatifida* have complex structures, with different proportions of sugar linkages (1,3-linked fucose, and 1,3-, 1,4-, and 1,6-linked galactose), as well as sulphate substitution patterns (2- or 4-position of fucosyl residues, and 3- or 6-position of galactosyl residues) (Hemmingson, Falshaw, Furneaux, & Thompson, 2006). Because of their varied biological properties, there is significant interest in synthesizing glycoconjugates from fucoidan-binding proteins for use in biological studies. For example, complexes of fucoidan with complement protein exhibited anti-complementary activity in the classical pathway (Tissot et al., 2003). However, reports from the standpoint of food systems are scarce. Thus, this study aimed to prepare BSA–fucoidan conjugates via the Maillard reaction under dry-heat conditions and to investigate their molecular characteristics.

2. Materials and methods

2.1. Materials

BSA was purchased from Sigma Aldrich (St. Louis, MO, USA). Fucoidan that had been extracted from the sporophyll (Miyeokgui) of cultured Korean *U. pinnatifida* (Miyeok) was acquired in a dried state from HarimBio Co., Ltd. (Wando, Korea). The chemical composition provided by the manufacturer was as follows: 97.9% fucoidan (polysaccharide + sulphate group) and 27.5% sulphate group. All the other chemicals used in the present study were of analytical grade.

2.2. Preparation of BSA–fucoidan conjugates

BSA and fucoidan (at a 1:3.6 M ratio of BSA to fucoidan) were mixed together in deionized water, using a magnetic stirrer at room temperature for 2 h. The completely dissolved sample solution was then freeze-dried. A desiccator containing saturated KBr was first placed in the oven at 60 °C for 30 min to achieve equilibrium temperature and relative humidity (RH). The freeze-dried samples were then placed in the dessicator and incubated at 60 °C and 79% RH for 96 h, after which they were lyophilized and stored at −18 °C before further analysis.

2.3. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to the method of Laemmli (1970), using 4–12% polyacrylamide gradient Bis-Tris gels (Invitrogen, Carlsbad, CA, USA). The samples were heated at 70 °C for 10 min in a 62.5 mM Tris–HCl sample buffer (pH 6.8) containing 2.5% SDS and 14.3 mM β-mercaptoethanol. An aliquot (10 μl) of the sample solution was then loaded into each well, and two pieces of gels were run at the same time at a constant voltage (200 V) for 35 min, using NuPAGE MES SDS running buffer (20×) (Invitrogen). After electrophoresis, one gel was stained with Coomassie brilliant blue G-250 for protein determination, and the other with the GelCode glycoprotein staining kit (Pierce Biotechnology, Rockford, IL, USA) for carbohydrate determination.

2.4. High-performance size-exclusion chromatography

High-performance size-exclusion chromatography (HPSEC) was performed using two SEC columns (TSK G3000 PWxl and TSK G2500 PWxl, 7.8 × 300 mm; Toso Biosep, Montgomeryville, PA, USA), equipped with a UV detector at 280 nm (Waters 2487; Waters Corp., Milford, MA, USA), a refractive index detector (Waters 2414), a multi-angle laser light scattering (MALLS) detector (HELEOS; Wyatt Technology Corp, Santa Barbara, CA, USA), a pump (Waters 510), and an injector valve with a 200 μl sample loop (model 7072; Rheodyne, Rohnert Park, CA, USA). The column was equilibrated and eluted with 0.15 M $NaNO_3$ containing 0.02% NaN_3 at a flow rate of 0.4 ml/min. The samples were dissolved in distilled water and filtered with a cellulose acetate membrane (3.0 μm-pore size; Whatman International Ltd., Maidstone, UK) before injection. The protein concentration was 4.0 mg/ml and the molecular weight values were calculated, using ASTRA version 5.3 software (Wyatt Technology Corp.).

The number of fucoidan molecules (N) glycated to one BSA molecule was calculated using the following formula, as described by Liu and Zhong (2013):

$$N = (M_{w2} - M_{w1})/M_{w3} \quad (1)$$

where M_{w1} is the average molecular weight of monomeric BSA, M_{w2} is the average molecular weight of the conjugate, and M_{w3} is the average molecular weight of fucoidan.

2.5. Atomic force microscopy

The morphologies of native BSA, the BSA–fucoidan mixture, and the BSA–fucoidan conjugates were imaged by atomic force microscopy (AFM), using the 5500 AFM instrument with PicoView version 1.6.4 software (Agilent Technologies, Santa Clara, CA, USA). All the samples were diluted to 10 ppm protein using deionized water, and 2 μl of each sample were spread onto a freshly cleaved mica substrate and air-dried for more than 2 h prior to imaging.

2.6. Particle size measurements

The particle size distribution of the samples was measured by using a Nanotrac Ultra dynamic light scattering (DLS) system (Microtrac Inc., Montgomeryville, PA, USA). Briefly, the mean particle size was characterised in terms of the mean surface area diameter, which was defined as $MA = \Sigma V_i/\Sigma(V_i/d_i)$, where V_i is the volume percent between sizes and d_i is the size represented by the centre between any two sizes.

2.7. Intrinsic fluorescence emission spectroscopy

Intrinsic emission fluorescence spectra of the samples were obtained on a SpectraMax M2^e device (Molecular Devices LLC, Sunnyvale, CA, USA). Sample solutions (1 mg/ml of protein) were prepared in 10 mM phosphate buffer (pH 7.0). The solutions were excited at 279 nm, and emission spectra were recorded from 300 to 400 nm at a constant slit of 10 nm. All the determinations were conducted in triplicate.

2.8. Circular dichroism spectroscopy

Samples were prepared at a protein concentration of 1 mg/ml, and the spectrum of distilled water was used as a control. Circular dichroism (CD) spectra were recorded in the far-UV region (180–260 nm), using a Chirascan plus device (Applied Photophysics, Leatherhead, UK) at 25 °C. Data were expressed as the mean residue ellipticity (θ) in deg cm^2 dmol^{-1}. The α-helix content of each sample was computed, using the following equation, as described by Morrisett, David, Pownall, and Gotto (1973):

$$\%\ \alpha\text{-helix} = ([\theta_{222}] - 3000)/(-36000 - 3000) \quad (2)$$

where $[\theta_{222}]$ is the molar ellipticity value at 222 nm.