Food Hydrocolloids 71 (2017) 1–7

Contents lists available at ScienceDirect

Food Hydrocolloids

journal homepage: www.elsevier.com/locate/foodhyd

Synthesis and structural characterization of lysozyme–pullulan conjugates obtained by the Maillard reaction

Long Sheng, Peng Su, Ke Han, Jiahui Chen, Anqi Cao, Zhilong Zhang, Yongguo Jin, Meihu Ma*

National Research and Development Center for Egg Processing, College of Food Science and Technology, Huazhong Agricultural University, Wuhan, Hubei 430070, PR China

ARTICLE INFO

Article history:
Received 16 December 2016
Received in revised form
13 April 2017
Accepted 21 April 2017

Keywords:
Lysozyme
Pullulan
Maillard reaction

ABSTRACT

The purpose of this study was to produce lysozyme-pullulan conjugates under mild Maillard reaction conditions and investigate the influence of conjugation on structural and physical properties of the glycation product. The covalent attachment of pullulan to lysozyme was confirmed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The optimum condition chosen for glycation was 60 °C at pH 7.5 for 5 days with a lysozyme to pullulan molar ratio of 1:8. Fluorescence and circular dichroism (CD) spectrum analysis suggested remarkable changes in the structure and conformation occurred to the lysozyme. The average particle size of glycosylated lysozyme was 3.5-fold to the native lysozyme and 2.4-fold to the heated lysozyme ($P < 0.05$). The surface hydrophobicity was greatly reduced and the solubility of lysozyme at different pH solutions was improved after covalent binding with pullulan ($P < 0.05$). Meanwhile, lysozyme-pullulan conjugates showed a broader range of antimicrobial activity than native lysozyme.

1. Introduction

Lysozyme (EC 3.2.1.17), also known as muramidase or N-acetylmuramide glycanhydrolase, is an ubiquitous enzyme that could damage Gram-positive bacterial cell walls by catalyzing hydrolysis of 1,4-α-linkages glycosidic between N-acetylmuramic acid and N-acetyl-d-glucosamine residues found in the peptidoglycan layer of bacterial cells (Bayarri, Oulahal, Degraeve, & Gharsallaoui, 2014; Ma, Tang, Yin, Yang, & Qi, 2013; Sagermann & Matthews, 2002). Lysozyme is widely distributed in plants and animals, and the egg white is a rich natural source of lysozyme, which constitutes 3–4% of the total egg white proteins (Abeyrathne, Lee, & Ahn, 2013). Lysozyme has a low molecular weight (14.3 kDa) and a high isoelectric point (pI = 11.16) (Kuehner et al., 1999). Lysozyme is a single chain protein that belongs to the class of globular proteins. It contains five α-helical regions and five β-sheets regions, linked by β-turns and random coils (Prilusky et al., 2011). The structural, physicochemical, and biological properties are well characterized, which makes lysozyme a model protein for biochemical applications and food systems.

Most recently there has been great focus on Maillard reaction, a natural and nontoxic method of modifying protein, to improve the protein functionality, such as solubility, foaming properties, gel properties, emulsifying properties and heat stability (Kim & Shin, 2016; Liu, Ru, & Ding, 2012). Compared to mono-, di- and oligosaccharides, polysaccharides possess larger molecular weight and less reducing groups (Chen et al., 2013). Therefore, polysaccharides were generally chosen to glycosylate protein to limit the extent of Maillard reaction and refrain from generating advanced glycation end products, deep color and unacceptable aroma in the later stage (Oliver, Melton, & Stanley, 2006). Lysozyme was reported to conjugate with xanthan gum under mild Maillard reaction condition (pH 8.5 and 60 °C for 10 days) and the conjugation exhibited broader antimicrobial activities as compared with native lysozyme (Hashemi, Aminlari, & Moosavinasab, 2014). Scaman, Nakai, and Aminlari (2006) found that dextran-conjugated lysozyme exhibited higher heat stability, pH and heat solubility and better emulsifying property than the unmodified lysozyme. However, study of glycosylated lysozyme by mean of Maillard reaction mostly focused on improving the function and is limited regarding the structural and conformational properties of protein.

Pullulan, produced by *Aureobasidium pullulans*, is widely applied

* Corresponding author.
E-mail address: mameihuhn@163.com (M. Ma).

http://dx.doi.org/10.1016/j.foodhyd.2017.04.026
0268-005X/

in the food and pharmaceutical industries because of its outstanding physical and chemical properties such as low viscosity, nontoxicity, odorless, tasteless, slow digestibility, high plasticity and edible (Sheng, Tang et al., 2016). Pullulan is a linear homopolysaccharide consists of glucosyl residues reduplicative units linked by α-1,4-linkages and α-1,6-linkages. This specific construction endows pullulan excellent solubility and extremely low viscosity compared to other polysaccharides (Dixit, Mehta, Gahlawat, Prasad, & Choudhury, 2015). On the other hand, pullulan molecules behave as flexible random coils and have net charge in water (Jiang, Wu, & Kim, 2011). The viscosity of pullulan solution does not change with heat, pH, and most metal ions including sodium chloride (Prajapati, Jani, & Khanda, 2013). All of these factors indicate that pullulan could become the most widely used biopolymer in a large number of technological platforms. Pullulan might be used for Maillard reaction to expand proteins applied in complicated food system especially at isoelectric pH, high salt concentrations, and high temperature. To our best knowledge, no details of the conjugation of proteins with pullulan have been carried out to date.

Based on the discussions outlined above and potential applications of the conjugation of protein with polysaccharide, the aim of this research was to investigate the optimal condition of the reaction for the highest yield of lysozyme-pullulan conjugates. Meanwhile, the changes in structural and physical of lysozyme were also studied for in-depth insight into the mechanism of Maillard reaction.

2. Materials and methods

2.1. Materials

Lysozyme from chicken egg white was purchased from Sigma (St. Louis, MO, USA). Pullulan with an average molecular weight of 20 kDa was obtained from Hayashibara Biochemical Laboratories (Okayama, Japan). All other reagents used in this study were provided from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China) and were of analytical grade.

2.2. Preparation of lysozyme-pullulan conjugates

Lysozyme-pullulan conjugates were prepared using Maillard reaction conditions, as reported previously (An et al., 2014). Mixtures of lysozyme-pullulan in various ratios (by weight) were dissolved in 50 mM sodium phosphate buffer solution (pH 7.0). Sodium azide (0.02%, w/w) was added to prevent bacterial growth. The sample solutions were stirred on a magnetic stirrer at room temperature for 2 h to completely dissolve the mixture. The pH values of the solutions were adjusted by carefully adding 0.1 N HCl or 0.1 N NaOH to the desired pH. Solutions were freezed-dried and then put into a desiccator containing saturated KBr solution at the bottom and incubated at 60 °C at 79% relative humidity. The samples with lysozyme alone were also treated in the same way for comparison. Triplicates were carried out for each experiment.

2.3. SDS-PAGE

SDS gel electrophoresis was performed in 1.0 M Tris−HCl buffer (pH 6.8) for stacking gel (5% acrylamide) and 2.0 M Tris−HCl buffer (pH 8.8) for separating gel (12% acryl−amide). Migration was performed at 80 V in the stacking gel and at 120 V in the separating gel, in 25 mM Tris, 192 mM glycine and 0.1% SDS, pH 8.3. About 10 μL protein sample (2 mg/mL) was loaded onto the gel. Standard proteins were used as a marker (10−170 kDa). After migration, gels were stained by classic solution (Coomassie Brilliant Blue R-250 0.1% in 25% ethanol, 8% acetic acid) for 30 min. Destaining steps were carried out by the immersion of gels in 10% acetic acid and 10% ethanol.

2.4. Measurement of UV-absorbance

The UV-absorbance of samples were measured according to the method of Lertittikul, Benjakul, and Tanaka (2007). Appropriate dilution (10−80 fold) was made using distilled water and the absorbance was measured at 294 using a spectrophotometer (NANO 2000C, Thermo, USA) for UV-absorbance.

2.5. Measurement of intrinsic fluorescence emission

Intrinsic fluorescence emission spectra were measured with a RF-5300 fluorescence spectrophotometer (Shimadzu, Japan). Sample (0.1 mg protein/mL, w/v) solutions were prepared in 50 mM phosphate buffer solution (pH 7.0). The excitation wave-length (slit width, 5 nm) was set at 280 nm and the emission wavelength range was from 300 to 420 nm, with a scanning speed of 1200 nm/min. The phosphate buffer solution (50 mM, pH 7.0) was used as the blank for all samples.

2.6. CD spectroscopy

CD of protein was investigated by a model J-1500 CD spectrometer (JASCO, Japan). The spectral bandwidth was set at 1 nm. The sample was solubilized in 50 mM phosphate buffer solution (pH 7.0) with the protein concentration of 0.2 mg/mL and then added to the quartz cell of 0.1 cm light path length. CD spectra were obtained from 190 to 250 nm at 25 °C.

2.7. Dynamic light scattering (DLS) measurements

Determination of the mean size hydrodynamic diameter of 100 μg protein/mL was carried out using dynamic light scattering spectrometer (Zetasizer Nano ZS90, Malvern Instrument Corp.) equipped with a He−Ne ion laser at a wavelength of 632.8 nm and a scattering angle of 90°.

2.8. Measurement of surface hydrophobicity

The hydrophobicity of the samples was measured according to the 1-anilinonaphthalene-8-sulfonic (ANS) method (Kato, Sasaki, Furuta, & Kobayashi, 1990) with slightly modified. Solutions of 100 μL ANS (0.3 mg/mL) was mixed with 2 mL protein (0.05 mg/mL, 0.10 mg/mL, 0.15 mg/mL, 0.20 mg/mL and 0.25 mg/mL). The samples were shaken on a vortex mixer for about 5 s and then standing for 30 min. Fluorescence intensity was measured at the excitation wavelengths of 370 nm with the emission wavelength scanning from 350 to 650 nm. The fluorescence intensity was measured as a function of protein concentration. The slope of the function was expressed as the surface hydrophobicity index (H_0).

2.9. Determination of solubility

Solutions with different pH at 5, 6, 7, 8, 9, 10, 11, and 12 were adjusted by 1 M HCl or 1 M NaOH. Lysozyme (1 mg/mL) was dissolved or dispersed in the above solutions by stirring for 2 h at room temperature. Then the protein solutions were centrifuged at 10000 *g* for 20 min at 4 °C. The protein content of the supernatants was measured the absorbance at 280 nm using a standard curve of lysozyme. In all cases, before absorbance was determined, protein solutions were diluted (1:1) with 8 M urea to prevent the interference of non-soluble solids. Solubility was expressed as the