International Journal of Biological Macromolecules 92 (2016) 600–606

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

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

The changes of secondary structures and properties of lysozyme along with the egg storage

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ARTICLE INFO

Article history:
Received 25 April 2016
Received in revised form 18 July 2016
Accepted 21 July 2016
Available online 22 July 2016

Keywords:
Lysozyme
Storage
Structure

ABSTRACT

The changes of lysozyme structure during egg storage were investigated by Fluorescence spectroscopy, Circular Dichroism spectroscopy and Fourier Transform-Raman spectroscopy, and the relationships between lysozyme properties (enzyme activity, emulsibility and foamability) and secondary structure were also discussed. During the storage, the percentages of α-helix and β-turn declined, conversely the β-sheet and random coil increased, the polarity of microenvironment around tryptophan residue gradually decreased. The results suggested that the conformation of lysozyme became more flexible during egg storage. Lysozyme activity was decreased but ESI and FA of lysozyme rose up with the storage duration. Correlation analysis revealed that second structure of lysozyme significantly affected lysozyme properties during egg storage. These findings would certainly be useful for further study of food storage and production.

1. Introduction

Lysozyme (EC 3.2.1.17), also known as muramidase or *N*-acetylmuramide glycanhydrolase, is a ubiquitous enzyme and its structure is highly conserved [1]. Lysozyme has a low molecular weight (14300 g/mol) and a high isoelectric point (pI = 11.16) [2]. Lysozyme contains five α-helical regions and five β-sheets regions, linked by β-turns and random coils [3]. There are 6 tryptophans and 3 tyrosines in lysozyme, and three of the Trp residues are located in the substrate binding sites, two are in the hydrophobic and one is located at the edge of lysozyme. Among them, Trp 62 and Trp 108 which are located in the substrate binding sites play important parts in binding to substrate and stabilizing the structure [4]. Lysozyme could damage Gram-positive bacterial cell walls by catalyzing hydrolysis of 1,4-β-linkages glycosidic between *N*-acetylmuramic acid and *N*-acetyl-D-glucosamine residues found in the peptidoglycan layer of bacterial cells [5,6].

Besides the antibacterial activity, lysozyme has also been reported to have functions including binding property, fungistatic activity, antitumor activity, inhibition of HIV replication and association with nucleic acid [7–11]. The lysozyme has been widely used in pharmaceutical industry and food industry as an anti-microbial agent and food preservative. 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 [12].

Egg traits can be significantly influenced by the length of storage period. Physicochemical changes of egg white happening was related to changes in egg white protein during storage [13,14]. Lysozyme is widely utilized as emulsifying and binding agent [15]. Change in lysozyme structure is at least one of the reasons responsible for the changes in the functional properties of lysozyme [16–18]. Unfortunately, information about changes in lysozyme structure and functional properties during egg storage is quantitatively limited. The study of molecular changes in lysozyme along with the egg storage and how the structure changes affect the functional properties of lysozyme will certainly be significant value in producing functional egg products and the further study of food storage. Furthermore, this study could supply new information for researching the relationships between the structure and function of protein during food processing.

In this research, the structure changes of lysozyme were evaluated by Fluorescence spectroscopy, Circular Dichroism (CD) spectroscopy and Fourier-Transform (FT) Raman spectroscopy during storage period. The protein functions such as emulsifying property, foaming property and lysozyme activity at different stages were investigated. Meanwhile, the relationships between

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http://dx.doi.org/10.1016/j.ijbiomac.2016.07.068
0141-8130/

the protein function and secondary structure of lysozyme were also analyzed by bivariate correlation analysis.

2. Materials and methods

2.1. Materials

Fresh chicken eggs (60 ± 0.5 g, average weight) from Hy-line variety Brown hens laid not over 24 h were collected from the Poultry Research Centre farm of Huazhong Agricultural University. All eggs were stored at 22 °C and 65% relative humidity for 50 days. Structure and other measurements were taken on 10 eggs per treatment every 10 days.

2.2. Extraction of lysozyme

Lysozyme was prepared according to reported method with a minor modification [19]. Egg white was manually separated from the yolk and pooled. Four hundred milliliters egg white were diluted with 3 vol of distilled water. The mixture was adjusted to pH 7.0 with 1 M HCl. The supernatant was collected by centrifugation (10000 × *g*, 10 min) at 4 °C. D152 resin (30% of egg white) was added to the supernatant. The mixture was stirred for 5 h, and discard the supernatant. An equal volume of 0.02 M (pH 6.5) PBS containing 1 M NaCl was added to the resin and stirred for 1 h. The supernatant was collected after standing. The supernatant was placed in an ultrafiltration cup, 30,000 ultrafiltration membrane was used for further purification. Ultrafiltration pressure was 0.2 MPa. Lysozyme fractions were desalted by diafiltration with deionized water at 4 °C for 2 days and freeze-dried. The purity of obtained lysozyme was determined by SDS-PAGE (date was not shown).

2.3. Fluorescence spectroscopy

Fluorescence measurements were obtained using an IF-5401 spectrofluorimeter (Hitachi, Japan) equipped with a xenon lamp source and a 1.0 cm quartz cell. The fluorescence emission spectra were recorded in the range of 300–500 nm upon excitation at 280 nm. The widths of both excitation slit and emission slit were set at 5 nm. Lysozyme solution (10 μg/mL) was prepared in 10 mM phosphate buffer (pH 7.2).

2.4. Circular dichroism (CD) spectroscopy

CD spectra were determined on a Jasco J-810 spectropolarimeter (Jasco, Japan) at wavelengths from 190 to 250 nm. Lysozyme (0.2 mg/mL) in 10 mM phosphate buffer (pH 7.2) was measured with a 1 mm cell in the far-UV region. Each spectra was obtained as an average of three scans to reduce noise before structure analysis was performed.

2.5. Fourier transform-Raman spectroscopy

FT Raman spectra were collected on an INVIA laser Raman spectrometer (Renishaw, UK) at room temperature. Lysozyme was prepared as a solid. Measurements were conducted under the following conditions: laser wavelength, 785 nm; grating density, 600 grades/cm; laser power, 25 mW; slit width, 200 μm; exposure time of 60 s. The protein spectra obtained were baselined, and the intensity was normalized using the phenylalanine peak at 1006 cm^{-1}. Gaussian was selected as input parameters for a least-squares curve-fitting procedure.

2.6. Determination of lysozyme acitivity

2.6.1. Microorganism

Micrococcus lysodeikticus 1.0634 was used in this study and stored at −80 °C in this laboratory.

2.6.2. Preparation of culture medium

The fluid medium included 5.0 g of beef extract, 10.0 g of peptone, 1.0 g of glucose and 5.0 g of NaCl in 1 L of distilled water. The medium pH was 7.5, and the medium was sterilized at 121 °C for 15 min.

The solid medium included 20.0 g of agar, 5.0 g of beef extract, 10.0 g of peptone, 1.0 g of glucose and 5.0 g of NaCl in 1 L of distilled water. The medium pH was 8.0, and the medium was sterilized at 121 °C for 15 min.

2.6.3. Preparation of the bacterium suspension

Cultures were inoculated into 250 mL flask that contained 50 mL of the fluid culture medium and subsequently incubated at 28 °C for 24 h with shaking at 200 rpm. Inoculation of three consecutive. Then a small amount of bacterial liquid transferred to the solid medium and incubated at 28 °C for 48 h. Thallus were removed by distilled water, and then centrifuged at 6000 rpm for 10 min. The sediment was washed with distilled water again. The collected cells was suspended in 0.1 M phosphate buffer (pH 6.2). The absorbance was measured at 450 nm and located at 0.75 ± 0.05.

2.6.4. Activity testing

One hundred microlitres lysozyme solution (50 μg/mL) was mixed with 900 μL bacterium suspension. The change of absorbance was monitored (450 nm) at different interval of time for up to 5 min. Activity of lysozyme solutions has been estimated from the decrease in absorbance value observed in the presence of active lysozyme due to lysis of the cell suspension. Experiments were performed in triplicate in each case.

2.7. Measurement of emulsifying properties of lysozyme

Emulsifying activity index (EAI) and emulsion stability index (ESI) of lysozyme were determined according to the method of Pearce and Kinsella [20]. To prepare emulsions, 40 mL of lysozyme solution (1 mg/mL) was mixed with 10 mL of corn oil. The mixture was homogenized using high-speed homogenizer (Hansen Co., Staufen, Germany) at the speed of 10000 × *g* for 1 min. Zero point one milliliters of the emulsion was immediately taken from the bottom of the container after homogenization, and diluted with 1 mg/mL SDS solution. The turbidity of the diluted emulsion was then measured at 500 nm using a spectrophotometer. ESI of lysozyme was estimated by measuring the turbidity of the emulsion at 500 nm first immediately after emulsion formation and then after keeping for 30 min. EAI (m^2/g) and ESI (min) values were calculated by the following equations:

$$EAI(m^2/g) = 4.606 \times DF \times A_0/(c \times a \times p \times 10,000)$$

$$ESI(min) = (A_0/A_0 - A_{30}) \times 30$$

Where DF is the dilution factor; p is the optical path (1 cm); a is the oil volume fraction in the emulsion (0.25); A_0 and A_{30} are the absorbance of the diluted emulsions at 0 and 30 min respectively; and c is the protein concentration in unit volume (g/mL) of protein in the aqueous phase. All treatments were performed in duplicate and mean was reported.