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Observations using Phosphorus-31 nuclear magnetic resonance (^{31}P-NMR) of structural changes in freeze-thawed hen egg yolk

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ABSTRACT

Hen egg yolk (EY) has a complicated structure consisting of lipids and proteins, and its structure is deeply related with its functional properties. ^{31}P-NMR is an efficient technique to non-destructively detect the dynamic behaviour of phospholipids, the main component of bio-membranes. We determined conditions for measuring the ^{31}P NMR spectra of EY and identified the components. ^{31}P-NMR was used to detect phosvitin, inorganic phosphate, and lipoprotein as well as structural changes such as granule collapse and freeze–thaw denaturation as signal changes. Freeze-thaw denaturation generated a new denaturation peak. We separated aggregates of LDL from freeze-thawed plasma using centrifugation. TEM and ^{31}P-NMR observations revealed that the denaturation peak corresponded to LDL aggregates. The ^{31}P-NMR spectra suggested the formation of multiple forms of LDL aggregates in which the head groups of phospholipid molecules adopt a face-to-face orientation, similar to that observed following the flocculation of lipoproteins or in the lamellar-like structures of phospholipids.

1. Introduction

Hen egg yolk (EY) has a very complicated structure consisting of numerous assemblies of lipids and proteins, and this complex structure is deeply related to its functional properties and utility, such as for emulsification and coagulation (Anton, 2013; Yang & Baldwin, 1995). Hen EY is composed of about 52% water, 31% lipids, 15% proteins, and the remaining constituents are carbohydrates, vitamins, and minerals (Guilmineau & Kulozik, 2006). The complex assemblies in EY mainly contain lipids and proteins, and interactions between phospholipids and proteins play essential roles in the structures and functionalities of these assemblies (Anton, 2013).

During various food manufacturing processes, EY is exposed to environmental changes such as heating, freezing, drying, the addition of salt or sugar, pH changes, and mechanical forces. These environmental stresses associated with pasteurization and preservation easily affect the functional properties of EY and consequently the quality of the products. Gelation of EY caused by freeze-thaw procedures is one example of an undesirable outcome which reduces the emulsifying properties of EY. Much research has therefore focused on developing new processing methods and new evaluation techniques to understand and control the denaturation of EY (Denmat, Anton, & Gandemer, 1999; Dou et al., 2017; Guilmineau & Kulozik, 2006, 2007; Sirvente et al., 2007; Strixner, Würth, & Kulozik, 2013; Telis & Kieckbusch, 1998; Wakamatu, Sato, & Saito, 1983).

Spectrophotometric techniques using nuclear magnetic resonance (NMR) non-destructively provide information regarding molecular structure and mobility. NMR techniques have recently been applied to food to analyze the mechanisms of structural changes in complex food systems (Au, Acevedo, Horner, & Wang, 2015; Au, Wong, & Acevedo, 2016; Hohmann et al., 2015), in addition to the qualitative and quantitative analyses.

^{31}P-NMR can be used to detect the molecular mobility of phospholipids, which are essential components of biological membranes. Phosphorus in the head group of a phospholipid is detectable with relatively high sensitivity and can easily provide information on molecular mobility because the magnetic anisotropic effect significantly affects the spectral width of the phosphorus signal. ^{31}P-NMR was mostly applied to simple systems 20–30 years ago. For example, Chiba et al. reported a relationship between emulsion stability and the headgroup motion of phospholipids (Chiba & Tada, 1989). Mine et al. studied the interactions between phospholipids, proteins, and fatty acids (Mine, Kobayashi, Chiba, & Masahiro, 1992), and structural changes in low-density lipoprotein (LDL) following the addition of phospholipase A2 (Mine, 1997). LDL forms 17–60 nm spherical particles with a core of triglycerides surrounded by a single layer of phospholipids and proteins (Anton et al., 2003). Furthermore, Kamo et al. reported interactions between phospholipids and neutral lipids (Kamo et al., 2003).

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The complex system of hen EY has been studied using ^{31}P-NMR, and phosphate, phospholipid and phosvitin signals were identified (Burt, Jeffreys-Smith, & London, 1986). Yanagisawa et al. reported ^{31}P-NMR spectral changes after salt addition and after freeze-thaw denaturation of EY samples containing salt two-times-diluted with deuterium oxide (D_2O) (Yanagisawa et al., 2009). They reported the spectral changes after salt addition and freeze-thaw denaturation might be the denaturation of LDL. However, the complex structure of EY presents challenges in identifying each peak in diluted, salted, freeze-thawed samples, and in elucidating the correlations between spectral changes and structural changes. Most researchers agree that freeze-thaw denaturation is mainly due to LDL aggregation of the plasma fraction (comprising mainly LDL and the water soluble protein livetin), although the mechanism of denaturation remains controversial (Kurisaki, Kaminogawa, & Yamauchi, 1980; Telis & Kieckbusch, 1997; Wakamatu, Sato, & Saito, 1982). Au et al. recently reported granules comprising aggregates of high-density lipoprotein (HDL) and phosvitin, and that these granules participate in freeze-thaw gelation during long-term freezer storage (Au et al., 2015). We envisioned that clarifying the correlations between ^{31}P-NMR spectra and the structures of EY components would facilitate the development of a new analytical method for evaluating the denaturation of EY.

2. Materials and methods

2.1. Egg yolk sample preparation

Fresh whole eggs were purchased from a local retailer in Tokyo and yolk samples were prepared using a modification of the method of Powrie, Little, and Lopez (1963) within five days of the packing date. Yolk was manually separated from albumen and chalazae. The yolk vitelline membrane was cleaned by rolling it on a filter paper. The yolk with the vitelline membrane was perforated with a Pasteur pipette and the yolk was poured into a tube.

Four yolk sample conditions were studied using ^{31}P-NMR: raw, raw with added salt (hereinafter called 'salted'), diluted with ultra-pure water, and salted and diluted. Raw samples were directly placed in an NMR tube (5 mm o.d.) using a needle with an outer diameter of 4.0 mm. Sodium chloride (NaCl, 10% w/w, 1.71 mol L^{-1}) was added to raw samples, then carefully mixed with a spatula to prepare the salted samples. An equal volume of ultra-pure water was added to aliquots of the raw and salted samples, then the samples were stirred using an EYELA cute mixer CM-1000 at 1500 rpm for 30 min to prepare the diluted, and salted and diluted, samples.

2.2. Plasma and granule preparation

Egg yolks were separated using the method of Mc Bee and Cotterill (1979) with a minor modification. Egg yolk was diluted with an equal weight of ultra-pure water, then mixed with a vortex mixer for several minutes and centrifuged at 16,000 × *g* for 30 min at 4 °C. The supernatant (unsalted plasma) was separated from the sediment (unsalted granules). Plasma samples were directly used for the ^{31}P-NMR measurements. NaCl (10% w/w) was added to the plasma samples to generate salted plasma. Granule samples were diluted with ultra-pure water to the same solids content as the plasma samples (about 1 g/mL). NaCl (10% w/w) was added to obtain salted granule samples. All samples were placed in NMR tubes using with a needle with the outer diameter of 4.0 mm.

2.3. Reagents and standard samples

D_2O and phosphoric acid were purchased from Kanto Chemical. Phosvitin and phosphatidylcholine (PC) from egg yolk were purchased from Sigma-Aldrich. All reagents were used without further purification.

2.4. Identification of EY components

Phosvitin (10 mg) was added to 2.0 g salted and diluted EY and stirred using the mixer for 30 min. Phosphoric acid aq. (1.0 g, 20 mM) was added to 1.0 g raw EY and 1.0 g salted and diluted EY, then the samples were stirred using the mixer for 30 min. Non-salted plasma was added to a final concentration of 25, 50 and 75% (w/w) to the salted and diluted EY samples. PC (24 mg) was added to 2.0 g diluted EY and to salted and diluted EY, then the samples were stirred using the mixer for 30 min.

2.5. Freeze-thaw denaturation process

2.5.1. Freeze-thaw denatured EY

EY samples (20 mL) in 50 mL tubes were stored at −35 °C for 15 h and thawed at ambient temperature. This process was repeated three times, then four sample (raw, salted, diluted, and salted and diluted) were prepared from the denatured EY.

2.5.2. Freeze-thaw denatured plasma and granule

Plasma (2.00 g), and granules (1.90 g) diluted with ultra-pure water to the same solids content as the plasma samples (about 1 g/mL), were each placed in 15 mL tubes and stored at −35 °C for 0, 60, 120, or 180 min. The samples were thawed at ambient temperature. The freeze-thawed plasma samples were directly placed in NMR tubes. NaCl (0.10 g) was added to the freeze-thawed granules and the samples were carefully mixed with a spatula. Aggregates of LDL were obtained by storing the plasma samples at −35 °C for 20 h and thawing at ambient temperature, and repeating once. The denatured plasma was centrifuged at 16,000 × *g* for 60 min at 4 °C.

2.6. ^{31}P-NMR measurements

^{31}P-NMR measurements were performed at room temperature on a JEOL ECA-600 spectrometer at 243.0 MHz using the following conditions: 45° pulse, 16 K data points, 90,000 Hz spectral window, 15 rotations/s spin rate, 1000 scans, and 4.5 s pulse delay in the presence of a proton decoupling field. Before sample measurements, D_2O was used to lock and shim the instrument. H_3PO_4 aq. (85%) in a capillary was used as an external standard to set 0 ppm.

2.7. Transmission electron microscopy (TEM)

TEM was used to confirm states of denaturation peak of freeze-thawed plasma sample comparing to ^{31}P-NMR results. The denatured plasma samples were centrifuged (16,000 × *g* for 60 min at 4 °C). It allowed to obtain a gelled material (upper layer) and translucent yellow liquid (lower layer). A droplet of various sample suspensions (raw plasma, freeze-thawed plasma, gelled components (upper layer after centrifugation), translucent yellow liquid (lower layer after centrifugation) were individually placed on carbon-film with a 400 Cu grid for 2 min and excess liquid was blotted by touching a corner of the grid with a filter paper. After partial drying, each sample was negatively stained with 2% (w/v) uranyl acetate for 2 min, then dried on the grid at room temperature. These samples were observed using a JEOL JEM-1200EX TEM operated at 80 kV.

3. Results and discussion

3.1. ^{31}P-NMR spectra of hen egg yolk

^{31}P-NMR can be used to detect several EY components, including the phosphoprotein phosvitin, inorganic phosphate, and lipoproteins such as LDL and HDL which mainly contain phospholipids, as reported by Burt et al. (1986). We initially conducted ^{31}P-NMR measurements of four EY samples (raw, salted, diluted, and salted and diluted) to

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