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Study on aggregation behavior of low density lipoprotein in hen egg yolk plasma by asymmetrical flow field-flow fractionation coupled with multiple detectors

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ABSTRACT

In this study, asymmetrical flow field-flow fractionation (AF4) coupled online with UV, multiangle light scattering (MALS), and fluorescence (FS) detectors (AF4–UV–MALS–FS) was employed for separation and characterization of egg yolk plasma. AF4 provided separation of three major components of the egg yolk plasma i.e. soluble proteins, low density lipoproteins (LDL) and their aggregates, based on their respective hydrodynamic sizes. Identification of LDL was confirmed by staining the sample with a fluorescent dye, Nile Red. The effect of carrier liquids on aggregation of LDL was investigated. Collected fractions of soluble proteins were characterized using sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE). Moreover, the effect of heat and enzymatic treatment on egg yolk plasma was investigated. The results suggest that enzymatic treatment with phospholipase A_2 (PLA_2) significantly enhances the heat stability of LDL. The results show that AF4–UV–MALS–FS is a powerful tool for the fractionation and characterization of egg yolk plasma components.

1. Introduction

Hen egg yolk is extensively used as an essential ingredient in a variety of food emulsions such as mayonnaise and salad dressings. It's components contribute to the formation and the stability of these emulsions by creating an interfacial film at the surface of dispersed oil droplets (Vincent, Powrie, & Fennema, 1966). However, egg yolk is still used rather empirically and physical properties of egg yolk-based emulsions are not entirely understood, as egg yolk is a complex mixture of several surface active components. Egg yolk can be divided into a plasma fraction, composed of soluble proteins (livetins) and low density lipoprotein (LDL), and a granule fraction, composed of phosvitin and high density lipoprotein (HDL) (Martin, Tattrie, & Cook, 1963). By studying competitive adsorption of proteins from hen egg yolk during emulsification, using two-dimensional polyacrylamide electrophoresis and mass spectrometry, it was shown that the proteins from LDL and HDL adsorb preferentially over the plasma proteins (Nilsson, Osmark, Fernandez, & Bergenståhl, 2007). The preferential adsorption of the lipoproteins has also been shown in other studies (Le Denmat, Anton, & Beaumal, 2000; Martinet, Saulnier, Beaumal, Courthaudon, & Anton, 2003; Mine, 1998b). The relative importance of LDL and HDL is, however, somewhat disputed. Several studies have focused on emulsification with LDL (Le Denmat et al., 2000; Mine, 1998a). LDL is considered to be a spherical colloidal particle with an inner part, consisting of triacylglycerides and cholesterol and an interfacial layer of phospholipids and proteins. Diameters between 17 and 60 nm have been reported from electron microscopy micrographs (Anton et al., 2003; Martin, Augustyniak, & Cook, 1964). Upon adsorption at interfaces, the LDL structure has been shown to collapse, resulting in a spreading of aggregate components at the oil–water interface (Martinet et al., 2003). By comparing the adsorption of vesicles, vesicles with incorporated protein and native LDL it was shown that the vesicles with protein adsorb more rapidly at an interface and that the behavior of the protein containing vesicles was similar to LDL (Anton, 2006). Hence, the proteins seem to play an important role for the adsorption and, thus, emulsification. Anton and coworkers purified LDL from egg yolk by gel filtration and found LDL to contain five major apoproteins (Anton et al., 2003).

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While the effect of pH, ionic strength, processing and enzyme treatment on emulsion properties of egg yolk has been studied (Daimer & Kulozik, 2008; Guilmineau & Kulozik, 2006a, 2006b; Le Denmat et al., 2000; Mine, 1998a), not much focus has been put on the effect of these parameters on LDL structure. One of the important functional properties of egg yolk plasma is its ability to undergo aggregation upon heat treatment, which plays an important role in the textural properties of final food products (Speroni et al., 2005). Heat stability is often desirable in emulsions, for instance to have the possibility to pasteurize them to assure microbiological safety etc. In order to improve the heat stability of egg yolk the enzyme phospholipase A_2 (PLA_2) can be used (Dutilh & Groger, 1981). The enzyme removes the acyl group in position 2 of the phospholipid, converting it to lyso-phospholipids (Mine, 1997). It is not totally understood how the increased heat stability is achieved but one suggested explanation is that lyso-phospholipids and/or free fatty acids interact with the proteins and protect them from denaturation (Mine, 1997).

In order to fully understand why the properties of an emulsion change, it is important to understand how the emulsifier is affected by environmental and processing conditions. Thus, detailed characterization of egg yolk plasma both in its native state and after different processes may, hence, increase the understanding of its functionality in emulsions. Asymmetrical flow field-flow fractionation (AF4) has attracted increasing interest in recent years owing to its broad dynamic separation range (approximately from 1 nm to well above 1 μm) and the utilization of an "open channel" void of stationary phase or packing materials (Giddings, 1993; Wahlund & Giddings, 1987). The absence of a stationary phase makes AF4 a gentle fractionation technique with limited shear and mechanical stress applied on sample components, particularly suited for the analysis of delicate analytes (such as, proteins and DNA) with full preservation of their native properties (Kim, Lee, Lim, & Moon, 2013; Williams, Runyon, & Ashames, 2010). Another main advantage of AF4 is the wide choice of carrier liquid which allows for the analysis of a sample in the formulation buffer. Owing to aforementioned advantages, AF4 can operate under conditions close to the native properties of biosamples, thus selected AF4 fractions can be collected and further analyzed with uncorrelated techniques such as microscopy or bioassays. Furthermore, AF4 can be coupled with a series of detectors (such as multiangle light scattering, MALS) to obtain more information on samples in a single run (Dou, Zhou, Jang, & Lee, 2014; Runyon, Nilsson, Alftrén, & Bergenståhl, 2013). Despite the versatility of AF4 for the study of complex biosamples and a range of food macromolecules and dispersions (Nilsson, 2013), AF4 technique has, to our knowledge, not yet been applied to egg yolk. At present, cryo-transmission electron microscopy (Cryo-TEM) and quasi-elastic light scattering (QELS) have been used for the size determination of egg yolk plasma components (Anton, 2013; Speroni et al., 2005; Yohannes et al., 2010). Cryo-TEM provides information on both the morphology and the size distribution of the sample at the same time. QELS is easy to use and a fast analysis. However, the size distributions from both techniques may not be highly reliable or reproducible for the samples of high polydispersity, especially for the presence of aggregated structures (Dou et al., 2013).

In the present study, we use AF4 coupled to a train of three detectors, namely, UV, MALS, and fluorescence (FS), to separate and characterize egg yolk plasma. The aim of this study is to evaluate the applicability of AF4 for the efficient and fast separation and characterization of egg yolk plasma, and to study the aggregation behavior of LDL in egg yolk plasma. The method can then be further used to study how different parameters such as changes in the environment and processing affect the properties of the plasma components.

2. Materials and methods

2.1. Materials

Nile Red, imidazole, sodium chloride (NaCl), potassium chloride (KCl) and calcium chloride ($CaCl_2$) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydrochloric acid (35–37%) and sodium hydroxide were purchased from Samchun Pure Chemical Co., Ltd. (Pyeongtaek, Korea). Deionized water was obtained from a Milli-Q Plus Ultra-Pure Water system (Millipore, MA, USA). All chemicals were of analytical grade and were used without further purification.

2.2. Preparation of egg yolk plasma

Fresh hen eggs were purchased from a local supermarket (Korea). The eggs were manually broken, and the albumen was discarded. The egg yolks were carefully rolled on a filter paper to remove albumen adhering to the vitellin membrane (Anton et al., 2003). This membrane was then punctured to flow out unspoiled egg yolk. Ten egg yolks were pooled and gently mixed at 4 °C. The egg yolk plasma was prepared according to the method described by McBee and Cotterill (1979). 4 mL pooled egg yolk was diluted with an equal volume of a 0.17 M NaCl solution and stirred with a magnetic stirrer for 1 h at 4 °C. The sample was then centrifuged at 10000*g* for 30 min at 4 °C, and the supernatant (plasma) was separated from the sediment (granules). The supernatant was centrifuged twice under the same conditions for a complete separation of plasma and granules. In order to study the effect of salt on preparation of egg yolk plasma, pooled egg yolk was also diluted with equal volume of carrier liquid 2 or 3 (as shown in Table 1). The pH of carrier liquid was adjusted by adding 1 M HCl. The calcium concentration of egg yolk was determined to be 5 mM by using a PHM82 pH meter with a model 93-20 calcium electrode (Orion research, Inc., MA, USA).The pH of egg yolk was determined to be 6.2.

2.3. Heat and enzymatic treatment of egg yolk plasma

1 mL of egg yolk plasma solution, prepared with carrier liquid 3 (Table 1) by the aforementioned method, was added into a centrifuge tube, and subjected to heat treatment in a water bath at 60 and 65 °C, respectively. Once the desired heating time was reached, the tube was immediately cooled in ice-water to room temperature. Subsequently, the heat-treated egg yolk plasma solution was analyzed by AF4. Enzymatic treatment of egg yolk plasma was carried out with phospholipase A_2 (PLA_2). The mixture of 1 mL egg yolk plasma solution, prepared with carrier liquid 3, and 1 μL PLA_2 solution (provided by Källbergs Industri AB, Töreboda, Sweden) was incubated in a water bath at 40 °C for 2 h and then cooled immediately in ice-water to room temperature. In order to study the effect of enzymatic treatment on the heat stability

Table 1
Main salts and pH values of egg yolk and carrier liquid.

	Main salts	pH value
Egg yolk	Ca^{2+} 5 mM, 40 mM NaCl and 53 mM KCl	6.2
Carrier liquid 1	10 mM imidazole and 40 mM NaCl	7.0
Carrier liquid 2	10 mM imidazole, 45 mM NaCl and 53 mM KCl	6.2
Carrier liquid 3	10 mM imidazole, 40 mM NaCl, 53 mM KCl and 5 mM Ca^{2+}	6.2