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Influencing emulsifying properties of egg yolk by enzymatic modification by phospholipase D from *Streptomyces chromofuscus* Part 1: Technological properties of incubated egg yolk

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ABSTRACT

Impact of a direct incubation of egg yolk with phospholipase D (PLD) from *Streptomyces chromofuscus* was investigated with respect to its effect on rheological and emulsifying properties. Egg yolk has been shown to be a suitable substrate for incubation with PLD after a slight dilution with water (70/30, w/w). A considerable increase of egg yolk viscosity during incubation was observed correlating with formation of phosphatidic acid due to enzymatic activity. Emulsions prepared with such an incubated egg yolk showed a higher viscosity, in particular at high egg yolk concentration. Especially in the case of lower concentrations, smaller droplets indicating better emulsifying activities were detected in emulsions prepared with the incubated egg yolk compared to the application of untreated egg yolk. Stability after heat treatment of the emulsions could be significantly improved by the application of egg yolk incubated with PLD.

1. Introduction

Due to its excellent emulsifying properties egg yolk is of high importance for food industry. Technological characteristics of egg yolk are determined by its specific composition, *inter alia* proteins and phospholipids. Egg yolk mainly contains approx. 50% water, 32% lipids, and 16% proteins [1] and can be separated into a water insoluble granule fraction and a soluble plasma fraction [2]. Regarding the dry matter composition, granule fraction consists of 70% high density lipoproteins (HDL), 16% phosvitin, and 12% low density lipoproteins (LDL) which form the granule particles [3]. Plasma fraction consists of 85% LDL and 15% livetins (related to dry matter). LDL form spherical particles with a core of neutral lipids (approx. 64%) surrounded by a surface layer of apo-proteins (approx. 14%) and phospholipids (approx. 22%) [1,2]. Phosphatidylcholine (PC) with 73% and phosphatidylethanolamine (PE) with 15.5% of total phospholipids content represent the most important phospholipid fractions of egg yolk [4].

LDL spherical particles (LDL-SP) are assumed to mainly determine emulsifying activity of egg yolk [5]. During emulsification LDL-SP break-up at the oil droplet surface and neutral lipids diffuse into the oil phase [6]. Apo-proteins and phospholipids are adsorbed at the oil droplet surface [7]. The exact mechanism, e.g. whether the components adsorb at the oil droplet surface individually or as complexes, has not been completely clarified yet [8].

It is well known that emulsifying activity of egg yolk can be improved by modifying egg yolk phospholipids with phospholipases [9]. Treatment with phospholipase A_2 (PLA_2) which catalyzes cleavage of the acyl group at the sn-2 position of the phospholipid molecules improves heat stability of egg yolk containing emulsions [10].

Another possibility of phospholipid modification results from treatment with phospholipase D (PLD) hydrolysing the phosphate ester group of phospholipids [11–13]. Using egg yolk lecithin as substrate, mainly choline and phosphatidic acid (PA) are formed [14]. Additionally, PLD can cause a transphosphatidylation with other polar groups, e.g. alcohols, forming phospholipids with modified head groups [15,16]. Due to such an activity this enzyme has become relevant in the pharmaceutical industry during the last years [17].

PLD is an ubiquitous enzyme found in bacteria, fungi, plants and mammals. Especially PLD from *Streptomycees chromofuscus* is often used to investigate the behaviour of this type of phospholipases [18–20]. It has to be mentioned that its enzymatic activity depends on availability of calcium ions in the substrate solution [21,22].

However, mainly isolated phospholipids from soybean [16,20] or egg yolk [23,24] have been used as substrates, but not the whole

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egg yolk, so far. Therefore, data concerning hydrolysis of phospholipids by PLD in a complex food matrix and its effects on functional properties of egg yolk, e.g. emulsifying behaviour and heat stability, are not available yet.

Hence, a phospholipase D from *Streptomyces chromofuscus* was used for the direct incubation of egg yolk. This enzyme may by an alternative to application of PLA_2 in the food industry, because a bitter taste for such an incubated egg yolk caused by liberated unsaturated fatty acids was published recently [25].

Effects on technological properties as well as on egg yolk structure changes and on emulsions prepared with such an egg yolk were investigated. This paper will discuss the influence of incubation on technological properties. Changes of egg yolk structure as well as of emulsion properties will be described in a second publication [26].

2. Materials and methods

2.1. Materials

Liquid pasteurized egg yolk was obtained from local egg product manufacturer (Ovobest, Neuenkirchen, Germany). Phospholipase D from *Streptomycees chromofuscus* (PLD) and egg yolk phospholipid standards (PC, PE, and PA) were purchased from Sigma Aldrich (Steinheim, Germany). Miglyol 812N, a caprylic/capric triglyceride, used for emulsion preparation was delivered from Caesar & Loretz (Hilden, Germany). Other chemicals (analytical grade) were obtained from Sigma Aldrich (Steinheim, Germany).

2.2. Enzymatic modification of egg yolk

Egg yolk diluted with deionized water (70/30, w/w) was applied for incubation with PLD. The amount of enzyme was 750 units per g phospholipids contained in diluted egg yolk. The reaction was carried out at 50 °C according to optimum enzyme temperature as specified by the manufacturer. In order to obtain a better distribution of enzyme and substrate, egg yolk was gently stirred. During incubation, samples of egg yolk were taken every 60 min, quickly frozen in liquid nitrogen and freeze-dried before analysis. Incubation of egg yolk which was used for preparation of o/w emulsions was stopped after 4 h by cooling down in iced water to 4 °C.

2.3. Isolation and quantification of phospholipids

Phospholipids were extracted from dried egg yolk using solid phase extraction (SPE) according to the method described by Descalzo et al. [27]. Dried egg yolk samples and a chloroform/methanol solution (2:1, v/v) were mixed in a lockable glass tube for 30 min. After separation of insoluble egg yolk components, the organic phase was transferred into a vial and evaporated under nitrogen. Dried lipid fraction was dissolved in chloroform and added to a normal-phase silica column (LiChrolut Si 60, 500 mg, Merck, Darmstadt, Germany). Phospholipid fraction eluted with methanol was concentrated using rotary vacuum evaporator and then the phospholipids were re-dissolved with chloroform.

Separation and quantification of individual phospholipids was carried out by a slightly modified high performance thin layer chromatography method (HPTLC) on Kieselgel 60 G, 20 cm × 10 cm (Merck, Darmstdt, Germany) according to Helmerich and Koehler [28]. A HPTLC set of Camag (Muttenz, Switzerland) was used for this analysis. 8 μl of the chloroform solution containing the phospholipids were applied to the HPTLC plates via Linomat 5 and plate development was performed by an aqueous solution of ethylacetate/chloroform/n-propanol/methanol/KCl (25/25/25/12/9, v/v/v/v/v) using a vertical chamber. Subsequently, plates were dipped in a copper sulphate pentahydrate/orthoposphoric acid detection reagent. Phospholipid spots were visualised by heating the plates on the TLC Plate Heater III at 180 °C for 10 min and quantified by densitometric evaluation using the TLC Scanner 3 at 400 nm. Concentrations of individual phospholipids were determined using the phospholipid standards at different dilutions.

2.4. Preparation of oil-in-water (o/w) emulsions

Untreated egg yolk as well as egg yolk being incubated with PLD were used for preparation of egg yolk suspensions standardised by egg yolk protein content (from 2% to 0.1%). The required amounts of egg yolk were dispersed in a 0.15 M NaCl-solution and stirred for 60 min with a magnetic stirrer at room temperature after adjusting to pH 7.0. Then, Miglyol was added to each suspension during 2 min under continuous stirring at 1800 min^{-1} to obtain a pre-emulsion containing 30% (w/w) of oil phase. Final homogenization was carried out using an Ultra-Turrax (IKA, Staufen, Germany) at 18,000 min^{-1} for 2 min at 20 °C. Afterwards, emulsions were cooled down in iced water to 7 °C.

2.5. Characterisation of dispersed state of emulsions

To characterise the egg yolk emulsification behaviour, particle size distribution in the emulsions was measured by laser diffraction using a Mastersizer X (Malvern Instruments, Malvern, UK) after diluting the emulsion 1:20 (w/w) with 1% sodium dodecyl sulphate (SDS) solution (for break-up of non-covalent bonds (protein aggregates)) and tetra-sodium pyrophosphate solution (1:20,000) [29]. The area-weighted average droplet diameter d_{32} was applied to characterize the dispersed state of the emulsion.

In order to analyse the flocculation behaviour of the oil droplets in the emulsions, an additional laser diffraction measurement was carried out without addition of SDS solution. The laser diffraction method not only detects oil droplets as single particles, but also aggregates of oil droplets or proteins in the continuous phase which were for example formed after heat treatment of the emulsions. Therefore, this measurement also detects aggregates of oil droplets as single particles. To characterise the aggregation of oil droplets, a flocculation factor as the ratio between particle sizes measured before and after addition of SDS solution to the emulsion was calculated [30]. Higher values of this factor indicate a higher degree of flocculation whereas a value close to 1.0 shows that there was negligible flocculation in the emulsion.

2.6. Heat treatment of egg yolk stabilised emulsions

Thermal treatment of emulsions for heat stability evaluation was carried out at 75 °C. 15 ml of emulsion were filled into glass tubes (15.5 cm long and 1.5 cm inner diameter). A pre-heating step was carried out in a water bath with a temperature of 80 °C for 3 min to accelerate the heating process. Then, the glass tube was transferred into a second water bath with the treatment temperature of 75 °C and held for 7 min. After finishing heat treatment, emulsions were transferred into an iced water bath and cooled down to 7 °C. Particle size distribution of the heat-treated emulsions was measured 24 h after the treatment.

2.7. Rheological characterisation of egg yolk and emulsions

Apparent viscosity of the diluted egg yolk during incubation with PLD was monitored using rotational viscometer Rheo-Logic MC 10 (Anton Paar, Stuttgart, Germany) with standard cylinder system Z2 (DIN 53019). 60 ml of the diluted egg yolk were transferred into a rheometer cup immediately after addition of PLD. Apparent viscosity was measured for 6 h at incubation temperature of 50 °C at a constant shear rate of 20 s^{-1}.