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Improved Approaches to Separate High-Value Phospholipids from Egg Yolk

by

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Abstract

Egg yolk contains approximately 10% (w/w) phospholipids (PL), of which about 70% is phosphatidylcholine (PC). Conventional methods of PL extraction from egg yolk involve using hazardous organic solvents. Supercritical carbon dioxide (SC-CO_2), as a "green" solvent, had been applied for the extraction of PL from egg yolk but resulted in limited success due to low recovery and purity of the final extracted PL.

Hydrolysis of egg yolk after soluble proteins removal, referred to as pellet, by a combination of protease and Lipase AY30 reduced emulsion stability evident by larger oil droplets size and higher coalescence index. A cream fraction obtained from Protease P and Lipase AY30 treated egg yolk pellet was subjected to PL extraction using SC-CO_2 in the presence of 8% ethanol as a co-solvent. The enzymatic treatment with Protease P and Lipase AY30 significantly improved the recovery of PC from 47% in dry yolk to 85%, 70% and 61% for dry, intermediate-moisture cream (20%), and “as is” cream (45% moisture), respectively, based on initial yolk weight. A higher purity of PC and PL (84% and 103%) was obtained by using hydrolysed pellet with the intermediate moisture content (20%), compared to dried egg yolk sample.

Low-density lipoproteins (LDL) contain about 90% of PL from egg yolk. Possible interaction between egg yolk components and polysaccharides can be a potential technique for LDL separation. A simple method was proposed to isolate LDL from egg yolk using 0.2% to 0.6% xanthan gum at egg yolk natural pH. The mechanism of LDL separation with xanthan gum was suggested to be a

combination of different interactions such initial electrostatic and hydrophobic forces and physical properties of the polysaccharide and its complex with egg yolk LDL such as shear thinning behaviour of xanthan gum solution and density difference of LDL-xanthan gum complexes. PL is a high-value component from egg yolk with potential applications in pharmaceutical and nutraceutical industries. Developing methods to improve the recovery and purity of PC and PL from egg yolk while eliminating hazardous organic solvents use will help to protect the environment and enhance food safety.

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Table of Contents

1. Introduction and thesis objectives 1

1.1. References 6

2. Literature review 10

2.1. Egg yolk composition and its bioactive components 10

2.1.1. Phosvitin 12

2.1.2. IgY 13

2.1.3. Sialic acid 13

2.1.4. Phospholipids 14

2.2. Emulsion systems 15

2.2.1. Emulsifying compounds 16

2.2.1.1. Phospholipids 16

2.2.1.2. Proteins 22

2.2.2. Egg yolk emulsifying properties 26

2.2.2.1 pH and ionic strength 27

2.2.2.2. Thermal effect 29

2.2.2.3. Enzymes 30

2.2.2.4. Other processing factors 32

2.2. Lipid extraction and fractionation 33

2.3.1. Organic solvent extraction 34

2.3.2. SC-CO_2 extraction 36

2.3.3.Biopolymer interactions as an approach for lipid separation 41

2.4. References 44

3. Physicochemical properties of leftover egg yolk after livetins removal……..…58

3.1 Introduction 58

3.2. Materials and Methods 60

3.2.1. Egg yolk fractionation 60

3.2.2. Preparation of reconstituted egg yolk 61

3.2.3. Characterisation of egg yolk fractions 61

3.2.3.1. Proximate analysis 61

3.2.3.2. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) for determination of protein profile 62

3.2. 4. Emulsifying properties 63

3.2.4.1. Oil droplet size, flocculation index (FI) and coalescence index (CI).. 63
3.2.4.2. Zeta potential (ζ)..... 64
3.2.4.3. Surface hydrophobicity 64
3.2.5. Unadsorbed protein profile of emulsions from intact egg yolk..... 65
3.2.6. Rheological measurements 65
3.2.7. Statistical analysis..... 66
3.3. Results and discussion 66
3.3.1. Proximate composition of egg yolk and pellet 66
3.3.2. Protein profile of egg yolk fractions..... 67
3.3.3. Emulsion properties 70
3.3.4. Rheological properties of intact egg yolk and pellet..... 75
3.3.4.1. Strain sweep 75
3.3.4.2. Viscosity..... 77
3.3.4.3. Time sweep 78
3.5. Conclusions..... 80
3.6. References..... 81
4. Effect of enzymatic treatments on physicochemical properties of leftover egg yolk pellet after IgY separation 85
4.1. Introduction..... 85
4.2. Materials and Methods..... 87
4.2.1. Egg yolk pellet preparation..... 87
4.2.2. Enzymatic treatment 87
4.2.3. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) 90
4.2.4. Particle size distribution of pellet hydrolysates 90
4.2.5. Cream separation and determination of total lipid and protein 91
4.2.6. Phospholipids analysis..... 91
4.2.6.1. Sample preparation..... 91
4.2.6.2. Phospholipids quantification 92
4.2.7. Emulsion preparation and characterisation..... 93
4.2.8. Statistical analyses 93
4.3. Results and discussion 93
4.3.1. Hydrolysate properties..... 93
4.3.1.1. SDS-PAGE profile of leftover egg yolk pellet and its hydrolysates.. 93
4.3.1.2. Particle size distribution of yolk, pellet and pellet hydrolysates..... 94

4.3.1.3. Cream composition 97
4.3.2. Emulsion properties of hydrolysates 104
4.4. Conclusions 107
4.5. References 108
5. Effect of enzymatic hydrolysis on the extractability of phospholipids from leftover egg yolk using supercritical CO_2 111
5.1. Introduction 111
5.2. Materials and Methods 115
5.2.1. Egg yolk pellet preparation 115
5.2.2. Enzymatic treatment and cream preparation 116
5.2.3. Proximate analyses 116
5.2.4. SC-CO_2 extraction process 116
5.2.5. Phospholipids analysis 119
5.2.5.1. Sample preparation 119
5.2.5.2. Phospholipids quantification 120
5.2.6. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) 121
5.2.7. Statistical analyses 121
5.3. Results and discussion 122
5.3.1. Composition of dry yolk, pellet and creams 122
5.3.2. SDS-PAGE profile 123
5.3.3. SC-CO_2 extraction curves 124
5.3.4. Phospholipids content of the extracts 127
5.4. Conclusions 131
5.5. References 132
6. Moisture impact on extractability of phospholipids from egg yolk after enzymatic hydrolysis 136
6.1. Introduction 136
6.2. Materials and Methods 138
6.2.1. Egg yolk pellet preparation and enzymatic treatment 138
6.2.3. Proximate analyses 138
6.2.4. SC-CO_2 extraction process 138
6.2.5. Phospholipids analysis 139
6.2.6. Particle size distribution of pellet hydrolysates 140
6.2.7. Optical microscopy 140

6.2.8. Cryo-Scanning Electron Microscopy 140
6.2.9. Statistical analyses 140
6.3. Results and discussions 141
6.3.1. Composition of fresh cream and yolk 141
6.3.2. SC-CO_2 extraction curves 142
6.3. Phospholipids content 145
6.3.1. Recovery based on extraction feed material 145
6.3.2. Yolk-based recovery 151
6.4. Conclusions 153
6.5. References 153
7. Interaction between egg yolk and ionic polysaccharides: a potential approach for isolation of low density lipoproteins 156
7.1. Introduction 156
7.2. Materials and Methods 159
7.2.1. Materials 159
7.2.2. Sample preparation 159
7.2.3. Phase separation behaviour 160
7.2.4. Lipid content determination 160
7.2.5. Zeta potential (ζ) 160
7.2.6. Particle size distribution 161
7.2.7. Confocal laser scanning electron microscopy (CLSM) 161
7.2.8. Scanning electron microscopy (SEM) 162
7.2.9. Protein profile 162
7.2.10. "Pure" LDL preparation 162
7.3. Results and discussion 163
7.3.1. Effects of polysaccharide type and its concentration on particle size of egg yolk 163
7.3.2. Effect of pH on egg yolk/polysaccharide suspensions at 0.4% concentration 164
7.3.2.1. Zeta potential (ζ) 164
7.3.2.2 Particle size 167
7.3.2.3. Phase separation behaviour 168
7.3.3. Characterisation of cream phase of xanthan gum/yolk suspension 170
7.3.3.1. Protein profile of fractions from yolk-xanthan gum interactions at pH 6 170

7.3.3.2 Microscopic analyses of cream derived from yolk-xanthan gum 172
7.4. Proposed interactions between egg yolk and xanthan gum..... 174
7.5. Conclusions..... 178
7.6. References..... 178
8. Conclusions and recommendations..... 181
8.1. References..... 188

List of Tables

Table 3.1. Moisture, lipid and protein contents (%) of fresh yolk and leftover egg yolk. 66

Table 3.2. Proteins and their related molecular weights for intact egg yolk, leftover pellet and the supernatant. 69

Table 3.3. Emulsion properties (n=8) of yolk, pellet and reconstituted yolk in terms of oil droplet size, flocculation index (FI) and coalescence index (CI). 71

Table 4.1. Protein, lipid and protein content as well as PC, PE and LPC (%w/w) in the pellet, cream and subnatant from enzymatic hydrolyses of the pellet…….99

Table 5.1. Total lipid and major PL classes content (g/100g dry matter) of material were used for SC-CO_2 extraction……..122

Table 5.2. The recovery and purity of PC and PL (PC+PE+LPC) of different treatment (yolk -based). 129

Table 6.1. Total lipid and major PL classes content (g/100g dry matter) in yolk and cream were used for SC-CO_2 extraction. 141

Table 6.2. The recovery of PC and PE (cream-based) and purity of PC and PL for different treatments..... 147

List of Figures

Figure 2.1. Structures of the major egg yolk phospholipids. 17

Figure 2.2. Lipid shape and super molecular organization (polymorphism).. 18

Figure 2.3. A schematic of the four PL studied by Wu et al. (2000). 19

Figure 2.4. Carbon dioxide pressure-temperature phase diagram…….....38

Figure 2.5. A schematic representation of possible structural rearrangement upon mixing two different biopolymers……..43

Figure 3.1. General scheme of egg yolk fractionation for IgY isolation and pellet formation……60

Figure 3.2. SDS-PAGE profile of egg yolk and its fractions (pellet and plasma) and unadsorbed protein to oil droplet surface of egg yolk emulsions……...67

Figure 3.3. Strain sweep of yolk and pellet, G': Storage modulus (Pa), G": Loss modulus (Pa)……. 76

Figure 3.4. Egg yolk and leftover pellet shear viscosity at shear rates of 3-100 (S^{-1})……..……..78

Figure 3.5. Time sweep of yolk and pellet, G': Storage modulus (Pa), G": Loss modulus (Pa)……. …79

Figure 4.1. Schematic summary of experimental design…….…87

Figure 4.2. SDS-PAGE profile of the pellet and its hydrolysates……94

Figure 4.3. Particle size distribution profile of intact yolk, pellet and the hydrolysates of pellet…….....96

Figure 4.4. Phospholipids chromatograms of (PC: Phosphatidylcholine, PE: Phosphatidylethanolamine and LPC: lysophosphatidylcholine of yolk and creams obtained after digestion with Protease P, Protease M. …..……..……101

Figure 4.5. Recovery of two major PL classes (PC and PE) and total PL: (PC+PE) or (PC+PE+LPC) in the pellet and its creams obtained after enzymatic hydrolysis…….....103

Figure 4.6. Mean volume weighted diameter of oil dropltes of emulsions preparaed from the hydrolysates of six enzymatic treatments…….....104

Figure 4.7. Coalescence index of emulsions prepared from the hydrolysates ...105

Figure 5.2. Schematic presentation of whole process and SC-CO_2extraction....119

Figure 5.3. SDS-PAGE profile of the pellet and cream after hydrolyses with Protease P and Protease II……..…..124

Figure 5.4. Total extract yield trend of different treatments using the two-step SC-CO_2 extraction process……..….125

Figure 5.5. The CO_2 loading or slope of initial linear portion of extraction with neat CO_2 for dry yolk, pellet and creams……..…126

Figure 5.6. Chromatogram of PC, PE and LPC of extracts of yolk (solvent and SC-CO_2+ethanol) and cream (SC-CO_2+ethanol)……. ….. 128

Figure 5.7. PC recovery (cream-based) during the SC-CO_2+ethanol extraction step based on the amount of PC in the feed material used for extraction ……...131

Figure 6.1. Flow diagram of sample preparation and SC-CO_2 extraction……..139

Figure 6.2. Total extract yield trend (cumulative mass) of different treatments using the two-step SC-CO_2 extraction process……142

Figure 6.3. CO_2 loading (g extract/g CO_2) or slope of the initial linear portion of the extraction curves during the first step of extraction with neat CO_2……...145

Figure 6.4. a) Particle size distribution of egg yolk and cream (45% mositure) b) Light microscopy image of cream showing oil droplets……148

Figure 6.5. Cryo-SEM images of fresh egg yolk, fresh cream and dry cream structures (bar length: 10 µm).……149

Figure 6.6. The recovery of PC and total PL (PC+PE+LPC) of different treatments based on the original yolk (n=2). ……...….152

Figure 7.3. Average zeta potential (ζ) of suspensions prepared from egg yolk, *ι*-carrageenan/yolk, xanthan gum/yolk, gum arabic/yolk as a function of pH at fixed concentration of 0.4% of polysaccharides…….....….166

Figure 7.4. The average particle size of egg yolk suspension in the of 0.4% concentration of gum arabic, *ι*-carrageenan and xanthan gum at different pH levels……168

Figure 7.5. Phase separation trend for egg yolk dilution at 0.4% polysaccharide concentration and at pH 6……169

Figure 7.6. Lipid, protein and moisture contents in the cream obtained from xanthan gum/yolk suspensions at 0.2%, 0.4% and 0.6% xanthan gum concentration……....169

Figure 7.7. Protein profiles of different fractions of egg yolk (L) LDL (control) (C) cream (yolk/xanthan gum) (P) plasma (C2) cream from plasma/xanthan gum (S) subnatant-liquid from plasma/xanthan gum……...172

Figure 7.8. SEM micrographs for different fractions of egg yolk: intact fresh egg yolk , LDL , cream fraction from xanthan gum/yolk……. **...**173

Figure 7.9. Confocal micrographs of cream fractions obtained from egg yolk/xanthan gum suspension at pH 6…….**.....**174

Figure 7.10. Suggested mechanism behind LDL separation from egg yolk after xanthan gum addition to egg yolk…….**....**177

List of Abbreviations

AA	arachidonic acid
ANS	1-anilino-naphthalene-8-sulfonate
Apo-LDL	apoproteins of low density lipoprotein
SC-CO_2	supercritical carbon dioxide
CI	coalescence index
CL	cardiolipin
CLSM	confocal laser scanning electron microscopy
CMC	carboxymethyl cellulose
DPPC	dipalmitoyl phosphatidylcholine
DMPC	dimyristoyl phosphatidylcholine
DMPE	dimyristoyl phosphatidylethanolamine
DPPE	dipalmitoylphosphatidylethanolamine
DHA	docosahexaenoic acid
DSC	differential scanning calorimetry
DSPA	distearoyl phosphatidic acid
DPPA	dipalmitoyl phosphatidic acid
ELSD	evaporative light scattering detector
d3,2	surface weighted mean
d4,3	volume weighted mean
FI	flocculation index
GRAS	generally regarded as safe
G'	storage modulus
G"	loss modulus
HDL	high density lipoproteins
HLB	hydrophilic-lipophilic balance
n	flow behaviour index
IgY	immunoglobulin Y
LDL	low density lipoproteins
LPC	lysophosphatidylcholine

LPE	lysophosphatidylethanolamine
lyso-PL	lysophospholipids
PUFA	polyunsaturated fatty acids
NaSCN	sodium thiocyanate
Neu5Ac	acetylneuraminic acid
O/W	oil in water
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PI	phosphatidylinositol
PLA_2	phospholipase A_2
PLD	phospholipase D
PL	phospholipids
PS	phosphatidylserine
SAS	supercritical anti-solvent
SEM	scanning electron microscopy
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SM	sphingomyelin
SS	specific surface
So	hydrophobicity
TLC	thin layer chromatography
tan δ	loss factor
UPLC	ultra performance liquid chromatography
ζ	zeta potential

1. Introduction and thesis objectives

Choline is an essential dietary compound with critical function for the structural integrity of cell membrane, neurotransmission, lipid and cholesterol metabolism and transport and therefore for brain and liver function (Zeisel, 1992; Zeisel, 2000; Zeisel, 2006). The recommendations for Adequate Intake (AI) of choline required for adult humans are about 500 mg/day (Institute of Medicine, National Academy of Sciences USA., 1998) or higher for women during pregnancy and nursing. Recent studies show that current choline consumption is below dietary recommendations, and more effort is needed to promote the importance of this compound in human health (Duric et al., 2012).

Choline is a critical nutrient for brain and spinal system development of embryo and infants. Therefore, choline mostly in the form of phosphatidylcholine (PC) is routinely added to infant formulas (Institute of Medicine, 1998). Human milk is rich in choline; however, soy-derived infant formulas have lower concentration of choline (Zeisel, 2006). Most common sources of choline are organ meat such as liver and egg yolk. The only source of choline other than dietary intake is through *de novo* biosynthesis of PC (Zeisel, 2006). Only 33% of phospholipids from the soybean lecithin is PC, while egg yolk lecithin contains 66-76% PC. In addition to the considerable difference in choline concentration, phospholipids from egg yolk are characterised by the presence of long chain fatty acids (LC-PUFA) of the ω-6 and ω-3 family, mainly arachidonic (AA) and docosahexaenoic acids (DHA). These fatty acids are not present in soybean

phospholipids. Egg yolk phospholipids also contain sphingomyelin (SM), which is not present in phospholipids from plant sources.

Fresh egg yolk contains 10% phospholipids (PL) in its composition, of which 66-72% are PC (Palacios and Wang, 2005b; Anton, 2007a). Therefore, egg yolk PL are preferred over soy lecithin and routinely added to infant formulas. Egg yolk also contains a high concentration of immunoglobulin Y (IgY), at about 100-150 mg per egg. An average immunized hen can produce about 40 g of IgY per year, which is 10-15 times higher than the amount that can be obtained from the blood of animals like rabbits. Meanwhile, IgY, unlike animal antibodies does not show any cross-interaction effect (Kovacs-Nolan and Mine, 2004). Therefore, IgY has great potential as an economic source of oral vaccine for the treatment of bacterial and viral diseases such as diarrhea and fatal intestinal diseases and for the development of antibody against venoms of snakes, spiders etc. (Kollberg et al., 2003; Schade and Terzolo, 2006; de Almeida et al., 2008; Vega et al., 2012). IgY extraction from egg yolk can be beneficial from economic, animal welfare and medical perspectives. IgY can be easily separated from egg yolk through simple 10-fold dilution with water, leaving 90% of high-valued PL in the leftover egg yolk (Kwan et al. 1991).

Current techniques for PL extraction from egg yolk at industrial scale is not well defined, and mostly involves complicated processes, using several organic solvents such as hexane, acetone and diethyl ether (Schneider, 1989; Palacios and Wang, 2005a,b; Gadkowski et al., 2012). Due to the environmental, safety and quality issues such as the possible solvent residue in the final product,

new regulations require related industries to develop alternative techniques to reduce the amount of organic solvents used during the extraction process (Wu et al., 2009).

Supercritical carbon dioxide (SC-CO_2) as a "green" technology has shown great potential for the recovery of high value lipid products, with numerous plant extracts produced at industrial scale (Temelli, 2009). However, due to the presence of non-polar nature of SC-CO_2, slightly polar and polar compounds like PL cannot be recovered using neat CO_2 (Temelli, 1992; Catchpole et al., 2009; Temelli, 2009). Therefore, a small percentage of polar co-solvent addition is required to extract polar compounds. For food applications this solvent should be GRAS (generally regarded as safe); therefore, ethanol and water are the only solvents that can be considered as a co-solvent or entrainer (Catchpole et al., 2009; Temelli, 2009).

The possibility of PL extraction from egg yolk using SC-CO_2 technology had been investigated by others (Boselli and Caboni, 2000; Shah et al., 2004; Aro et al., 2009). Despite good achievements, the low recovery of PL, even in the presence of ethanol as a co-solvent has been a barrier for scale up purposes. It has been suggested that non-covalent bonds between apoproteins and PL in egg yolk structure is a barrier for the recovery of PL from egg yolk using SC-CO_2 (Burley and Vadehra, 1989). PL in egg yolk are complexed to apoproteins of lipoproteins. In addition, the presence of the strong emulsion structures of fresh egg yolk along with a high moisture content (50%) makes PL extraction a challenge.

Enzymatic aqueous extraction of oil from oilseeds has shown good potential for the recovery of oil from plant tissues without the use of organic solvents. Different types of enzymes have been applied to demulsify the emulsion formed during aqueous extraction of fruits and oilseeds (Domínguez et al., 1994; Sharma et al., 2002; Chabrand et al., 2008; Wu et al., 2009). However, the effect of enzymes on the extractability of PL from egg yolk has not been investigated. After enzymatic treatments, there is a possibility to break the bonds between PL and proteins, which may make the PL more accessible to SC-CO_2 and thus increase PL recovery.

Since egg yolk contains most of its PL in low density lipoproteins (LDL) particles (Anton, 2007b), the separation of these particles in an aqueous system using biopolymers such as polysaccharides can be another possibility for fractionation of high-value egg yolk components such as choline-rich LDL. Polysaccharides have been used extensively for the separation of bioactive proteins from dairy products and for the precipitation of lipids for IgY purification from egg yolk (Hatta et al., 1990; Ausar et al., 2001; Casal et al., 2006; Rojas et al., 2007). However, the potential of using different polysaccharides for the separation of individual LDL particles from egg yolk has not been reported.

Based on the above, it was hypothesized that through manipulation of egg yolk emulsion structure either through enzymatic treatments or through its fractionation by biopolymers such as polysaccharides in an aqueous system, high-value egg yolk components can be extracted through eco-friendly approaches. Therefore, the overall objectives of this thesis research were:

1. To understand the fundamental aspects of egg yolk behaviour under different treatments such as the removal of its soluble proteins or different enzymatic treatments, and

2. To apply these aspects to egg yolk processing to improve the PL extraction efficiency in terms of final product purity and PL recovery.

The specific objectives of this thesis research were:

1- To evaluate the physicochemical properties of egg yolk, including its emulsion structure and rheological properties after the removal of the majority of livetins (Chapter 3);

2- To investigate the effect of different enzymatic treatments on the emulsion properties and PL composition of leftover egg yolk (Chapter 4);

3- To assess the effect of leftover yolk hydrolysis with selected enzymes on the extractability of PL using SC-CO_2 with ethanol as co-solvent (Chapter 5);

4- To investigate the effect of structural changes and moisture content on the extractability of PL using SC-CO_2 (Chapter 6), and

5- To study the phase separation behaviour and potential separation of PL-rich LDL in diluted egg yolk through the possible interactions between selected anionic polysaccharides and egg yolk components (Chapter 7).

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2. Literature review

2.1. Egg yolk composition and its bioactive components

Fresh egg yolk contains about 50-53% dry matter, composed mainly of lipids (~65%) and proteins (~30%). Egg yolk provides all the nutrients required for the chicken embryo to develop. Lipids in egg yolk are all complexed through non-covalent bonds to apoproteins to form lipoprotein assemblies. The approximate composition of yolk lipids is about 62% triglycerides, 33% phospholipids (PL) and less than 5% cholesterol. Carotenoids, providing the egg yolk colour, make up less than 1% of egg yolk lipids (Anton, 2007a). All lipids in egg yolk are associated with lipoprotein assemblies. PL composition in yolk lipids is about 76% phosphatidylcholine (PC) and 22% phosphatidylethanolamine (PE). Other PL classes such as lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), sphingomyelin (SM), phosphatidylinositol (PI), phosphatidylserine (PS) and cardiolipin (CL) are found in minor amounts in egg yolk lipids. Polyunsaturated fatty acids (PUFA) represent 30-40%, while saturated fatty acids (SFA) make up about 45% of the total fatty acids of PL. Monounsaturated fatty acids (MUFA) account for 20-25% (Kuksis, 1992). PC has quite a high content of ω-3 and ω-6 fatty acids in the non-polar fatty acid chains and choline at the polar head, which gives PL from egg yolk a very high nutritional value (Kuksis, 1992). Proteins of egg yolk are present as free proteins like livetins or as apoproteins attached to lipids within low density lipoproteins (LDL) and high density lipoproteins (HDL).

Egg yolk major constituents based on dry matter are LDL (70%), HDL (16%), livetins (10%), phosvitin (4%) and other proteins (2%) (Anton, 2007a). LDL of yolk are spherical particles with an average diameter of 17-60 nm with a lipid core in liquid form (triglycerides and cholesterol esters) surrounded by a layer of PL and apoproteins. LDL are composed of 11-17% proteins and 83-89% lipids (Anton, 2007b). HDL have a diameter of 7-20 nm and contains 70-80% proteins and 20-25% lipids. Their lipids are made of about 65% PL and 30% triglycerides and 5% cholesterol. HDL are complexed to phosvitin through phosphocalcic bridges (Anton, 2007c).

Yolk is an emulsion. Egg yolk can be separated into two distinct phases of plasma and granules by ultracentrifugation or a simple two-fold dilution (Schmidt et al., 1956; Mcbee and Cotterill., 1979). Plasma, the aqueous phase, contains 77-81% of egg yolk dry matter and is composed of 73% lipids, 25% proteins and 2% ash. The major constituents of plasma are LDL (85%) and livetins (15%). Granules make up 19-23% of egg yolk dry matter and have very low solubility (20-30%). Granules are composed of 70% HDL, 16% phosvitin and 12% LDL (LDLg). At high alkaline or acidic conditions or ionic strength of over 1.7M NaCl disruption and solubilisation of granules occur, which may improve the emulsifying properties of the granules (Causeret et al., 1991).

About 50% of egg yolk proteins and over 90% of egg yolk lipids are in the plasma fraction, while granules account for 50% of egg yolk proteins and 7% of the yolk lipids (Saari et al., 1964). Therefore, from a technological point of view, the majority of PL will remain in the plasma fraction after two-fold egg yolk

dilution. However, at higher dilution factors, LDL from plasma co-precipitate with granules (Kwan et al., 1991). At 10-fold dilution, over 90% of egg yolk lipids remain with granules and this fraction is referred to as pellet. The aqueous fraction or the supernatant, composition is mainly livetins with a small amount of LDL (Kwan et al., 1991). Granules and plasma show distinct emulsifying properties, which will be discussed in more detail in the next section.

Yolk accounts for 36% of whole egg weight. Several compounds in egg yolk such as IgY, phosvitin and PL are well known and have been extensively studied for their great potential and applications as antioxidant, antimicrobial and nutritional compounds. The following is a summary of the functional and bioactive components found in egg yolk.

2.1.1. Phosvitin

Phosvitin is a phosphoglycoprotein. Nearly 50% of its amino acids is serine, of which 90% are phosphorylated. At neutral pH, phosvitin exists as random coil structure and it is highly charged (-179 mV) (Castellani et al., 2004; Anton et al., 2007c; Castellani et al., 2008). As a highly phosphorylated protein, phosvitin has strong chelating properties (Burley and Vadehra., 1989; Castellani et al., 2004; Guerin-Dubiard et al., 2007). Phosvitin also shows some bactericidal activity, which may correspond with its strong chelating activity (Khan et al., 2000). Excellent bonding capacity of the phosvitin to iron makes phosvitin a natural antioxidant for food and medical products (Lu and Baker., 1986; Lu., 1987; Nakamura et al., 1998; Lee et al., 2002; Ishikawa et al., 2004). Oligo-phosphopeptides derived from tryptic hydrolysis of phosvitin are reported to

enhance the bioavailability of calcium, which may find applications in nutraceutical products for the prevention of osteoporosis (Choi et al., 2005).

2.1.2. IgY

Livetins are fractions of α-, β-, γ-livetins in the proportion of 2:5:3, and all are water-soluble. The main component of α-livetin is an albumin while β- and γ-livetins are glycoproteins. α-Livetin has allergen property and γ-livetin, also called β-immunoglobulin or IgY is analogous to human antibody (Schade and Terzolo., 2006). IgY has been shown to have a number of antibacterial and antiviral properties based on *in vivo* and *in vitro* studies (Schade and Terzolo, 2006). Therapeutic effect of IgY against intestinal infections caused by *Escherichia coli*, *Helicobacter pylori*, *Salmonella*, *Rotavirus*, *Coronavirus* in human and animals has been demonstrated (Schade and Terzolo, 2006). IgY also has been shown to give positive results for the treatment of diseases like cystic fibrosis, celiac, colitis and dental plaque due to its antibacterial activity (Otake et al., 1991; Nilsson et al., 2008; Gujral et al., 2010). IgY antibodies have been developed as an economical source for preventing and treating reptile venoms, especially for economically challenged countries (de Almeida et al., 2008; Prabhu et al., 2010; Aurora et al., 2012). IgY can be extracted through simple dilution method from egg yolk. Further processing may be applied through chromatographic techniques to obtain high purity IgY.

2.1.3. Sialic acid

Sialic acid is the generic name for over 30 derivatives of neuraminic acid with an acyl group on the amino nitrogen, which are involved in cellular functions

and may be used as antiflammatory drugs. The most prevalent form of sialic acid in the nature is *N*-acetylneuraminic acid (Neu5Ac). Egg yolk contains Neu5Ac mostly in its yolk (0.2%), yolk membrane (1.8%) and chalaza (2.4%) (Koketsu et al., 1992; Seko et al., 1997; Hartmann and Wilhelmson, 2001).

2.1.4. Phospholipids

Egg yolk phospholipids (PL) contain an abundant amount of choline due to the presence of PC (66-76%). PC in egg yolk also has long chain polyunsaturated fatty acids (PUFA) including arachidonic acid (AA) and docosahexaenoic acid (DHA). However, soy lecithin is the most common commercial source of PL and PC makes up only 33% of the PL composition of soybean. Therefore choline content in soy lecithin is only a third of the choline content of egg yolk lecithin. In addition, soy lecithin also lacks ω-3 and ω-6 fatty acids. These fatty acids have a great impact on the growth and development of infants (Institute of Medicine, 1998; Gil et al., 2003). Choline plays an essential role in liver function, neuron system development and prevention of Alzheimer disease, cell membrane functionality, cholesterol metabolism and its reduction (Wright et al., 2004; Michel and Bakovic, 2007; Kuellenberg et al., 2012). In addition, PL from egg yolk have a broad application in pharmaceutical and food industries as emulsifiers and delivery systems.

Egg yolk PL is preferred over soy lecithin for parenteral nutrition applications due to some adverse reactions associated with emulsion stabilised with soy lecithin (Floyd, 1999; Rossi, 2007). Also, it has been reported that egg

yolk PL may have better oxidative stability than soy lecithin, which is preferred in food product applications (Palacios and Wang, 2005a).

2.2. Emulsion systems

An emulsion consists of two immiscible liquids (usually oil and water) with one liquid dispersed as small spherical droplets in the other by external forces like homogenization. A system that consists of oil droplets dispersed in an aqueous phase is called oil-in-water (O/W) emulsion. Emulsions are thermodynamically unstable systems because the contact between oil and water is thermodynamically unfavourable. To form a kinetically stable emulsion, stabilisers are required. Stabilisers can be classified as emulsifiers or thickening agents. Thickening agents mainly stabilise an emulsion by increasing the viscosity of the continuous phase. Whereas, emulsifiers are surface-active molecules, which can be adsorbed on the surface of droplets formed during homogenization. They form a protective membrane around the oil droplet that prevents the droplets from aggregation (McClements, 2005).

In practice, food emulsions are complex systems in which each phase and the dispersion can contain different compounds. Egg yolk is an example of an excellent emulsion and emulsifier system in nature. In egg yolk emulsion, granules are dispersed in the plasma fraction. The fact that egg yolk contains both proteins and PL, two major components necessary for emulsion stability, may spark the question which compound plays a greater role in yolk emulsifying properties. Proteins and PL are both amphiphilic, which means they can arrange

their hydrophilic and hydrophobic groups towards aqueous and oil phases, respectively, and stabilise emulsions.

In addition, charged PL and proteins can also stabilise emulsions through repulsive forces. However, proteins are large polymers, which can unfold, cover oil droplet surface and create an efficient viscoelastic layer around the oil droplets to enhance emulsion stability by steric forces. On the other hand, PL are small molecules and thus cannot solely cover the oil droplet to form a thick cohesive interfacial layer. Major contribution of PL to emulsion stability can be through incorporation into the interfacial phase and lowering of Gibbs free energy and interfacial tension due to their amphiphilic structure (McClements, 2005).

Regardless of the considerable differences between PL and protein structures, emulsifying behaviour and adsorption on the oil surface may change for each individual class of proteins and PL, depending on their structure and applied conditions such as pH (Handa et al., 1990). Since a major objective of this thesis is to understand the effect of egg yolk emulsion structure after enzymatic treatment on the extractability of PL, an introduction will be given on the effect of individual PL and proteins on emulsion stability and factors that may affect their emulsifying properties.

2.2.1. Emulsifying compounds

2.2.1.1. Phospholipids

The most common PL in egg yolk or soybean lecithin are zwitterionic PC and PE (McClements 2005) and their structures are shown in Figure 2.1. Based on their structure i.e. fatty acid chain and head group, PL represents a variety of

water binding capacity, imposing restrictions on their conformation and thus further interactions (Jendrasiak, 1996).

PC mainly forms a lamellar mesophase at ambient temperature in water, which can contain up to 40% of water, while its lyso-form makes it more hydrophilic and forms hexagonal I phase (Fig. 2.2) in water that may contain 55% water at 37 ^{o}C. PE forms both hexagonal II and lamellar phases (Fig. 2.2) in water (Spars and Krog, 2003). PC can adsorb more water than PE because the choline head group increases water affinity for PC.

Phosphatidylcholine **Phosphatidylethanolamine**

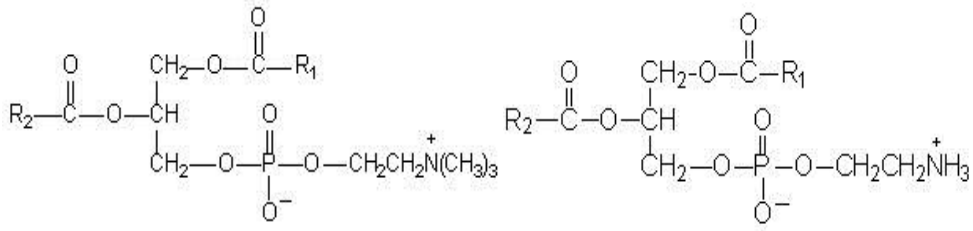

Figure 2.1. Structures of the major egg yolk phospholipids. R1 and R2 are fatty acid residues.

It is also evident that the addition of more double bonds in the hydrocarbon chain of PC increases the possible reactions with water molecules and the water absorption capacity of PC (Jendrasiak, 1996). Water binding capacity can affect protein-lipid interactions, and therefore it is an important factor on emulsion interfacial phase complex formation and stability (Escriba et al., 1997).

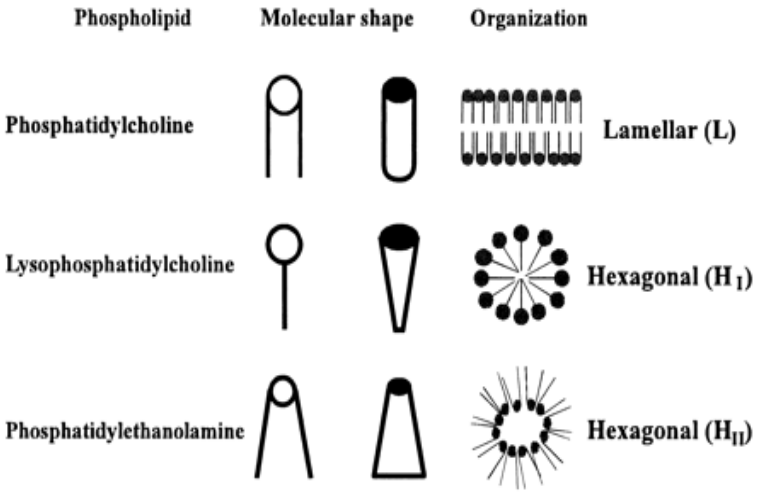

Figure 2.2. Lipid shape and super molecular organization (polymorphism). Image was reprinted from Escriba et al. (1997) with permission.

Wu et al. (2000) studied competitive absorption of four PL: dipalmitoyl phosphatidylcholine (DPPC), dimyristoyl phosphatidylcholine (DMPC), dimyristoyl phosphatidylethanolamine (DMPE), dipalmitoylphosphatidylethanolamine (DPPE) in a mixture with β-lactoglobulin, β-casein and human serum at the chloroform/water interface (Fig. 2.3). All PL were zwitterionic as DPPC and DMPC had a hydrophobic head group consisting of three methyl groups. They reported that PL head group structure had a greater impact on the equilibrium of interfacial tension than did the chain length. It appeared that DPPE and DMPE, which had smaller head groups, could pack as tightly as possible at the same bulk concentration; therefore, protein molecules could hardly occupy any interfacial space.

There are conflicting results regarding the emulsifying ability of different PL classes. Becher (1985) reported that both PC and PE are good O/W emulsifiers. Davis and Hansrani (1985) studied the coalescence behaviour of soy

oil droplets stabilised by the addition of PC, PE, LPC, PI, cholesterol, cholesterol oleate, PA and SM. Interfacial tension was significantly lower for emulsions prepared with LPC. In the same study, a mixture of PC and PE at different proportions (1:1, 1:2, 1:4) had no effect on interfacial tension.

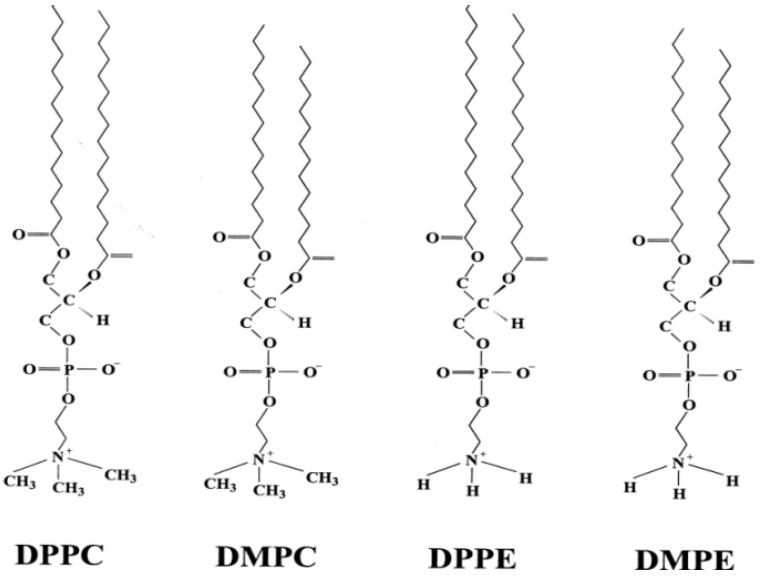

Figure 2.3. A schematic of the four PL studied by Wu et al. (2000). Image was reprinted from Wu et al. (2000) with permission.

Yeadon et al. (1958) reported that ionic PL such as PS and PA improved the stability of O/W emulsions due to higher electrostatic repulsion created by the negatively charged PL. PS and PA are negatively charged at pH 7 and despite the fact that they make only 1-2% of the total PL in egg yolk or soy bean, they confer a surface charge of about -40 to -60 mV (Washington, 1996).

Ishii and Nii (2005) studied the potential effect of hydrophilic and lipophilic groups of PL on O/W emulsion properties. A difference in the oil droplet size of emulsions was observed due to change in the hydrophilic and

lipophilic groups of PL. The authors reported that the oil droplet size of O/W emulsions prepared with a PC and PE mixture was mainly influenced by hydrophobic and hydrophilic groups of applied PL, thus hydrophilic-lipophilic balance (HLB) of PL was the main factor determining oil droplet size and emulsion stabilisation. In fact, an increase in carbon chain length of fatty acids can cause more hydrophobic interactions between side chains and the oil phase.

Natural crude PL, depending on their source, can have considerable differences in the chemical composition in terms of fatty acid chain length and saturation degree. This can cause some physicochemical and functional differences even within the same class of PL. Natural lecithin from soybean has no particular preference to water or oil with HLB values of about 8. Therefore, they may not be suitable for stabilizing O/W or W/O emulsions. Several chemical or enzymatic modification methods have been applied to increase the specific functionality of PL (McClements, 2005). There are different types of purified PL (Lipoid E80, Lipoid E100 , Ovothin 180, Ovothin 200) obtained from crude PL for emulsion and liposome applications. Crude PL have an iodine number of 60-80. Saturated PL are available in the market under the brand name of Lipoid E100-3 with an iodine number of 2-5 (Washington, 1996). Chemical or enzymatic hydrolysis of PL can drastically change their functional properties. The enzymatical conversion of PL to lyso-PL in order to increase its functional properties and emulsifying ability has been an approach taken by the food industry (Tirok et al., 2001; Le et al., 2007; Daimer and Kulozik, 2008; Buxmann et al., 2010a).

Interactions between proteins and polar lipids, like PL, are important from biological and technological aspects. Interactions between PL and proteins can be different based on the environmental conditions as well as PL head group and fatty acid acyl chain (Kristensen et al., 1997). Differential scanning calorimetry (DSC) results on β-lactoglobulin, distearoyl phosphatidic acid (DSPA) in an aqueous system indicated a specific interaction between phosphatidic acid (PA) and the protein. The same result was observed with dipalmitoyl phosphatidic acid (DPPA) and β-lactoglobulin, but not with PC and PE. Most probably, the charged head group of phosphatidic acid is essential for this interaction (Kristensen et al., 1997). Malmsten (1995) found out that there is no interaction between proteins and neutral lipids. Fatty acid chain length and its saturation is another factor influencing the interactions between proteins and PL (Redgrave et al., 1992; Kabalnov et al., 1995, 1996)

Kabalnov et al. (1995, 1996) showed that plasma lipoprotein stabilisation was affected by hydrocarbon chain composition of PC. Ibdah and Phillip, (1988) studied the effect of lipid composition and packing on apolipoprotein A penetration in monolayer lipid. Results proved that a higher saturation of fatty acid chain of PL could change its fluidity and decrease protein adsorption on surface. This evidence could also be true in emulsions and be reflected in interfacial film viscoelasticity and stability. Smiley and Richmond (2000) applied vibrational spectroscopic technique in order to understand the relationship between PC chain length and their assembly in the immiscible aqueous and

carbon tetrachloride (CCl_4) system. Results showed that PC with C16 and shorter chain fatty acid PC tend to form disordered layers.

Also, longer fatty acid chain length and its higher saturation degree can increase surface tension and promote hydrophobic interactions (Du et al., 1996). The saturation of acyl chain affects transition temperature and surface behaviour, which improves emulsion stabilisation (Washington, 1996). Martins et al. (2007) examined the adsorption of PC with different fatty acid chain lengths from C12 to C22. In PC with less than n <18, a complete desorption from the interface was observed, while there was no difference in interfacial energy for PC with n >18. Therefore, PL structure, including its polar head group and the composition of its fatty acids affects its emulsifying properties.

2.2.1.2. Proteins

The amphipathic nature of proteins can stabilise emulsions via a rearrangement and exposure of their hydrophobic and hydrophilic segments to the oil and aqueous phases, respectively, thus reducing interfacial tension in a manner similar to other surfactants and emulsifiers such as PL (Damodaran, 2005). In addition, as proteins are quite large molecules, they can stabilise emulsions through steric repulsion by forming a hydrodynamic barrier around the oil droplets (McClements, 2005). Protein adsorption followed by its unfolding and rearrangement on the oil surface causes some denaturation in the secondary and tertiary structures of proteins (Fang and Dalgleish, 1997). Therefore, protein structure after adsorption may not be necessarily similar to that of its parent one.

The effect of proteins on emulsion stabilisation is determined by internal and external factors. Number of amino acids, their nature and sequence, which in turn determine protein size, its structure and flexibility, are included as internal factors. External elements such as continuous phase pH, temperature, viscosity, the presence of other hydrocolloids and surfactants also significantly affect protein structure (Chen and Dickinson, 1998). In terms of internal factors, protein composition, resulting in the structure flexibility and unfolding capacity, is the primary determining factor of emulsion properties.

Water-soluble proteins generally have a higher percentage of charged segments and less hydrophobic groups. But, this cannot be necessarily true for all proteins. Two proteins with the same hydrophilicity and surface charge can show different solubility behaviour based on their amino acid composition and spatial arrangement within their tertiary structure. For example, serum albumin and myoglobin both have quite similar surface charge, but the degree of hydrophobicity is higher for serum albumin than that of myoglobin. Serum album is highly soluble even at its isoelectric point whereas myoglobin is insoluble at its isoelectric point (Lin and Zayas, 1987). Water solubility facilitates rapid diffusion of proteins to the oil droplet interface. A good balance of hydrophilic/lipophilic groups is necessary for a suitable emulsion capacity (Dickinson, 2003).

Flexibility can be defined as a protein's innate ability to undergo rapid conformational changes when it is transferred to different environments (Damodaran, 2004, 2005). Abundance of flexible patches in proteins allows easier unfolding and adjustment on interface. Unfolding is an important factor on protein

extension and its spreadability on the oil surface and therefore emulsion formation and stability. Molecules with higher molecular weight adsorb slowly but they can likely cover the interface better and form a thicker and viscous layer. Unfolding is a time dependent process affected directly by the original amino acid composition of the protein. Extensive intermolecular associations and strong bonds i.e. disulphide interactions limit unfolding capacity. Rigid proteins, with compact and highly ordered structure cannot perform this transition fast, thus they cannot decrease interfacial tension (Damodaran, 2005). Protein unfolding increases the number of contacts per protein molecule and makes desorption from the surface difficult. Casein due to its random structure can unfold and adsorb on the oil surface faster and mainly form a thin monolayer on the surface while globular proteins form a thick layer on the surface due to their more compact structure.

Some studies have shown that α-helix in the original secondary structure is more stable during interfacial adsorption. Higher concentration of proteins will decrease the protein adsorption due to large kinetic barriers. As a result, in order to cover more surface of oil droplet, proteins will form β-sheets (Sharma et al., 2002, 2010). Flexible proteins, sometimes called "soft" proteins, like casein, contain little secondary structure and unfold quickly at the surface, whereas in more packed globular proteins, unfolding takes longer (Dickinson, 1999). However, in general globular proteins form stronger and more viscoelastic films, which are quite resistant to coalescence (Wilde, 2000).

Emulsions can be destabilised through different mechanisms such as, flocculation, coalescence, creaming, Ostwald ripening and phase inversion. It is

worthwhile to have a brief look at the relationship between protein structure, external environmental factors and protein-stabilised emulsions. Oil droplets stabilised with a protein at a pH above or below the isoelectric point of the protein will repel each other and the emulsion will be electrostatically stabilised. Ionic strength or pH changes can lead to neutralization of the charge of droplets and promote flocculation. Some ions attaching to amino acids may change the protein conformation and steric repulsion (Dalgleish, 1997). Proteins form a viscoelastic polymer layer around the oil droplet. This layer creates a steric repulsion, which can also stabilise and increase emulsion stability.

It is interesting that sometimes addition of small amounts of surfactant into emulsions stabilised by proteins can destabilise these systems by diluting the protein concentration at the interface, displacing proteins or even changing their conformation (Fang and Dalgleish, 1997). High temperatures can impair protein emulsion properties by denaturing its structure. However, partial denaturation and unfolding under controlled heating conditions can expose more hydrophobic sites of proteins and increase hydrophobic interactions (Kim et al., 2002; Franco et al., 2000; Bengoechea et al., 2010).

Partial protein hydrolysis with enzymes such as trypsin, chymotrypsin and pepsin could improve emulsion properties of some dairy products due to the exposure of some functional groups (van der Ven et al., 2001). However, extensive protein hydrolysis forms small peptides, which are not able to form a good viscoelastic film and cover the oil droplet efficiently. Therefore, larger oil droplets will be formed that may promote further coalescence and oil separation.

2.2.2. Egg yolk emulsifying properties

Egg yolk is well known for its excellent emulsifying activity and has been applied extensively in different food products as an emulsifier. Most compounds in egg yolk i.e. proteins, PL, cholesterol and minor lipids have emulsifying properties. Egg yolk proteins have a wide range of molecular weight and isoelectric points, which can greatly contribute to the excellent emulsifying properties of egg yolk (Nilsson et al., 2007). However, they do not adsorb to the interface of oil droplets at the same level. Therefore, the adsorption of these compounds on the interface can be very competitive depending on the applied conditions such as pH and ionic strength. For example, phosvitin is an interfacial active protein; however, even after addition of pure phosvitin to egg yolk granules, HDL apoproteins could still displace phosvitin from the oil interface (Aluko and Mine, 1997).

There is a general agreement that apoproteins of LDL are the major contributors to egg yolk emulsifying properties (Vincent et al. 1966; Mizutani and Nakamura, 1985; Anton and Gandemer, 1997). Apoproteins of LDL are the most hydrophobic proteins and have a very flexible structure, making them highly surface active compounds (Mizutani and Nakamura, 1985; Vincent et al., 1966; Anton and Gandemer, 1997; Le Denmat et al., 2000; Dauphas et al., 2006). Nevertheless, depending on the applied conditions component properties and the emulsifying properties of egg yolk can change (Anton and Gandemer, 1999).

Heat, salt, pH, high pressure and to some extent enzymatic treatment are the major treatments applied in food processing, which can affect egg yolk

emulsion properties. Due to the complex structure of egg yolk, understanding its key emulsifying factors and the effect of applied conditions on the emulsifying mechanism is a challenging task. Therefore, researchers have tried to fractionate egg yolk into its plasma and granules to better understand the emulsifying properties of egg yolk. Different factors that impact these properties will be discussed below.

2.2.2.1 pH and ionic strength

Alteration of pH can change the conformation and solubility of proteins and their adsorption rate on the interface. Egg yolk proteins have different isoelectric points, which make their adsorption on the oil interface selective. For example, apoproteins of LDL as the major proteins of egg yolk have their isoelectric range between 6.5-7.3 (Kojima and Nakamura, 1985). Anton and Gandemer (1997) found that egg yolk had the highest emulsifying activity at pH 6 at which all egg yolk proteins except a polypeptide from LDL and phosvitin, were adsorbed on the oil interface. The best pH for livetins adsorption was at pH 6, whereas a very small amount of livetins was adsorbed at pH 3. Lower pH can degrade some egg yolk proteins before their adsorption at the interface, as Nilsson et al. (2007) reported significant degradation of some proteins at pH values below 4. Mine (1998b) found that the major proteins adsorbed at pH 7 and low ionic strength (0.1M NaCl) was HDL apoproteins from granules; neither phosvitin from granules nor livetins from plasma could be adsorbed on the oil interface. Granules at their native structure (neutral pH and low ionic strength) have low solubility and show poor emulsifying activity (Anton and Gandemer, 1997). However, it

was reported that disrupted granules in the presence of higher salt concentration (0.5M NaCl and pH 7) showed higher solubility and formed better emulsions compared to those of plasma and egg yolk (Le Denmat et al., 2000). Aluko and Mine (1998) found that egg granules formed more stable emulsions at pH 7 and pH 9 than those prepared at pH 4 and hypothesized higher stability of emulsions prepared with granules at alkali pH was due to the increased surface charge of phosvitin, which may help emulsion stabilisation through electrostatic repulsion.

Le Denmat et al. (1999) and Anton and Gandemer (1999) reported that pH 6 was suitable among the three tested pH levels (pH 3, 6 and 9) for fine emulsion formation in which all proteins except phosvitin could be adsorbed on the oil interface. They also reported that the most adsorbed protein at pH 3 was phosvitin, while it was not adsorbed at the interface at pH 6 and pH 9. It was believed that phosvitin's high net charge (-179 mV) at neutral and alkaline pH was the major reason for its drastic desorption behaviour. This was in contrast to the findings of Aluko et al. (1998) described above. PL in egg yolk are completely complexed to lipoproteins and they stabilise the LDL spherical structure. Therefore, during apoprotein adsorption there is a great chance for PL to adsorb at the interface since they are complexed with apoproteins. PL in yolk can contribute to emulsification through lowering the interfacial tension. Mine (1998 a,b) reported that PC and PE were adsorbed selectively at the oil droplet surface by pH alteration. The structural differences between PL components can alter their affinity to apoproteins at different pH levels. PC has a quaternary head group, which makes it a more basic compound compared to PE. At pH 4, where PL are

more negatively charged, the electrostatic interaction between the PC molecules and apoproteins are greater than those at highly alkaline pH. Such increased interactions enable PC molecules with a stronger quaternary base head group to bind more strongly to the proteins at the interface; whereas neutral pH favours PE adsorption at the oil surface.

Le Denmat et al. (2000) reported that PL concentration at the interface of emulsions prepared with plasma was affected by NaCl concentration but not by pH. They also found that PL were less concentrated at the interface with 0.15M NaCl concentration than 0.55M NaCl. In contrast, PL concentration at the surface for emulsions prepared with granules was sensitive to pH. The concentration of PL was very high at pH 3 and 0.55 M NaCl. Overall, these studies showed that both proteins and PL could be adsorbed selectively on the oil interface during emulsification depending on pH and ionic strength.

2.2.2.2. Thermal effect

Heat treatment, in order to eradicate pathogens in liquid yolk and its products like mayonnaise, is a critical process. Pasteurization is mainly performed at temperatures between 60 to 68 ^{o}C for 3.5 to 4.5 min. Egg yolk viscosity increases sharply at temperatures above 60 ^{o}C and its proteins start to coagulate at 70 ^{o}C. Some constituents of egg yolk can tolerate relatively higher temperatures. Le Denmat et al. (1999) showed that LDL and livetins of plasma were denatured at temperatures over 72 ^{o}C and 69 ^{o}C, respectively. Among HDL proteins, β-HDL could withstand quite severe temperatures; however, α-HDL started to degrade above 72 ^{o}C. Heat treatment above 72 ^{o}C did not affect α-phosvitin and

β-phosvitin. Overall, it seemed that emulsions prepared with heated granules showed excellent emulsion stability even after tolerating severe temperatures (75 ^{o}C).

Further study on the impact of heat treatment of granules demonstrate that LDL and α-HDL were sensitive to severe temperatures and β-HDL was more heat resistant (Anton et al., 2000). Native and disrupted granules showed similar values for creaming rate. Since considerable disruption of α-HDL in the granule native structure was observed, it was suggested that the phosphocalcic complex of granules did not have any function in terms of protection against high temperatures (Anton et al., 2000). Emulsions prepared with egg yolk that had been heated at 74 ^{o}C for a few minutes had slightly bigger oil droplet size than that of intact egg yolk; however, flocculation level decreased drastically (Guilmineau and Kulozik, 2006).

2.2.2.3. Enzymes

Enzymatic hydrolysis has been targeted as a potential tool for altering and improving egg yolk functional properties through industrial processes such as increasing heat tolerance during pasteurization or improving emulsion properties. After removal of lipids by organic solvents, the proteins showed poor emulsion properties; however, could be improved by controlled hydrolyses using several proteolytic enzymes (Guang and Wang, 2009).

Phospholipases gained attention because of their great impact on increasing heat stability of egg yolk and improved emulsion properties. Granules due to phosphocalcic bridges have very low solubility (~30%) at natural condition of egg yolk. Egg yolk solubility increased from 70% to 100% after hydrolysis

with Phospholipase A_2 (PLA_2) at egg yolk natural environment. Granules, after hydrolysis by PLA_2, were more heat resistant (Daimer and Kulozik, 2008). Mine (1997) investigated the effect of hydrolysis by PLA_2 on LDL structure. His conclusion was that a complex was formed between LDL apoproteins and lyso-phospholipids (lyso-PL) after LDL hydrolysis with PLA_2. Around 12% of egg yolk granules are heat sensitive LDL. It was suggested that the possibility of a complex formation between lyso-PL and LDL apoproteins could protect heat sensitive LDL proteins and improve overall heat stability of granules. It has been reported that the solubility of lyso-PL increased after treatment with PLA_2, which improved emulsion properties of egg yolk even after high temperature treatments (Daimer and Kulozik, 2008).

Phospholipase D (PLD) cleaves PL head group and forms phosphatidic acid, and lyso-PL, which has more hydrophilic behaviour. Emulsions prepared with egg yolk hydrolysed with PLD had small oil droplet size (Buxmann et al., 2010a). Structural changes of hydrolysed egg yolk with PLD before and after egg yolk heat treatment showed that about 50% of intact egg yolk structure was α-helix (Buxmann et al., 2010b). Intact egg yolk treatment with PLD significantly increased α-helix structure, while heating increased β-sheet and random structure, both in equal ratio. α-Helix followed by random coil was the most prevalent structure for enzymatically and heat treated egg yolk. Egg yolk incubated with PLD and then subjected to heat treatment contained a higher ratio of α-helix compared to egg yolk, which was only treated with heat. Anionic phosphatidic acid may interact with egg yolk proteins and change their β-sheet to α-helix,

which according to Anton et al. (2003) resulted in an increase in protein flexibility and improved emulsion stability.

Mine et al. (1993) reported that the presence of LPC increased α-helix and reduced β-sheet in the secondary structure of ovalbumin. However, such a structural change did not happen with PC. Scanning electron microscopy studies (Buxmann et al., 2010b) proved network formation and protein linking in egg yolk incubated with PLD. Therefore, increased viscosity can be attributed to the network formation in hydrolysed egg yolk structure. Emulsions prepared with PLD treated egg yolk had considerably higher surface charge. This suggested that phosphatidic acid has surface activity and could significantly contribute to the oil droplet interface. It seemed a complex was formed between egg yolk proteins and PL after digestion.

2.2.2.4. Other processing factors

Egg yolk is subjected to mechanical forces during the emulsification process employed for the manufacture of food emulsions such as mayonnaise. Anton and Gandemer (1997) and Aluko and Mine (1998) detected some HDL adsorption on the oil interface at pH 6, even though HDL are formed to be naturally complexed into granules and not to be adsorbed on oil interface after mechanical treatment. These authors suggested that slight mechanical disruption of granules during homogenization released some HDL from granule structure. Sirvente et al. (2007) showed that high pressure homogenization decreased protein solubility and created protein aggregates of LDL and livetins in plasma

fraction. Emulsion studies proved that even after LDL particles were disrupted by high pressure, their emulsion properties were similar to those of intact egg yolk.

Leftover protein fraction obtained after lipid extraction can be used as an emulsifying agent. The supercritical carbon dioxide (SC-CO_2) extraction technique has been applied to egg yolk for cholesterol-reduced egg production (Bringe et al., 1996). Cholesterol in contact with the oil phase can show interfacial activity through hydrophobic interactions (Jolivet et al., 2008). Whether cholesterol removal can change emulsifying properties of egg yolk and LDL of egg yolk was studied by Bringe et al. (1996) and later by Mine and Bergougnoux (1998).

Bringe et al. (1996) reported that emulsifying properties of low cholesterol egg yolk obtained after SC-CO_2 extraction was similar to those of intact native yolk. Mine and Bergougnoux, (1998) reported emulsions prepared with LDL after cholesterol removal had larger oil droplet size and less emulsifying activity. Important parameters such as extraction temperature, pressure, and the nature of oil used for emulsion preparation were different in these two studies. The presence of other proteins besides LDL apoproteins in total egg yolk after SC-CO_2 extraction could have a compensating effect for emulsifying activity.

2.3. Lipid extraction and fractionation

Total lipid extraction from oilseeds and other food matrices is based on the use of organic solvents such as hexane, chloroform, diethyl ether and others. Edible oil from oilseeds such as canola and soybean has been traditionally extracted using hexane at industrial scale. Environmental and safety concerns

oblige related industries to find alternative techniques for oil extraction. Aqueous enzymatic hydrolysis has gained attention as an eco-friendly technique to extract oil from oilseeds and plant tissues (Sharma et al., 2002; Chabrand et al., 2008; Wu et al., 2009; Towa et al., 2010).

Different types of enzymes such as pectinases and proteases have been used for disruption of plant cell walls or demulsification of creams obtained during the cold press or aqueous extraction of oil from oilseeds and fruits (Rosenthal et al., 1996). Regarding egg yolk, total lipid extraction has been limited to organic solvents for analytical scale application (Kuksis, 1992). Total lipid from egg yolk is usually extracted using a mixture of chloroform/methanol or hexane/isopropanol. For industrial scale, a clear and detailed procedure has not been defined. It seems at industrial level, egg yolk lipids are selectively fractionated through subsequent solvent extractions in order to isolate and purify PL from neutral lipids, as will be described in the following sections.

2.3.1. Organic solvent extraction

Egg yolk PL are commonly extracted using organic solvents through a series of complicated processes, but commercial scale PL extraction from egg yolk is not well described. Sim et al. (1994) used ethanol for fractionation of proteins and lipids using ethanol at 60 ^{o}C with ethanol-to-yolk volume ratio of 4:1. The aqueous ethanolic portion was stored at 2-4 ^{o}C for 12-16 h for neutral lipids to crystallize. After separation of crystals, containing cholesterol and triglycerides, the aqueous part was dried using a rotary evaporator. The dried solids were reported to have a purity of 89.1% PL with a recovery of 96.4%.

Juneja et al. (1994) used ethanol for PL extraction followed by using acetone for precipitation of PL at a large scale using 500 kg fresh yolk. The yield was 37.5 kg with 80% purity for PC. Quite a complicated protocol was described for industrial scale operation and used for PL separation through the European Commission. An intermediate lecithin containing 85% PL and a final fraction with 100% pure PL were obtained (EU Commission – Scientific Committee on Food, 1999).

Nielsen (2001) extracted PL from coagulated egg yolk by packing it in chromatographic tubes and performing consecutive extractions with acetone and ethanol. The elution from acetone extraction contained all triglycerides, cholesterol and 15% of PL of egg yolk. The subsequent elution with ethanol extracted the remained 85% of PL. It was claimed that the extract had high purity confirmed by thin layer chromatography (TLC); however, the exact purity was not reported. Later, Nielsen and Shukla (2004) described an *in situ* method for the extraction and purification of PL from spray dried egg yolk powder. The triglycerides in the ethanolic extract were removed by cold crystallization at 0 and -20 ^{o}C. About 80% of PL from dry yolk with a purity of 86% was recovered from the extraction. About 10% of cholesterol was co-extracted with PL in this procedure.

Using two different approaches, Palacios and Wang (2005b) investigated the impact of sequential lipid and PL extraction from fresh and heated yolks using different solvents such as ethanol, acetone, hexane, methanol and butanol. In the first approach, the recovery of PL from deoiled and not deoiled fresh and heated egg yolks using acetone and ethanol was studied. In the deoiled process,

extraction was applied to the PC-rich fraction obtained after deoiling of egg yolk with acetone, where the PC recovery of the final extract was about 60%. In the second approach referred to as "undeoiled procedure", total lipids were extracted by ethanol as described by Sim et al. (1991), and then PC in ethanol was precipitated with acetone. The highest purity of 55% was obtained for deoiled and heated egg yolk, which was significantly lower than the amount reported by Sim (1991). In a second study, Palacios and Wang (2005a) could achieve a high purity (95%) and recovery (96%) of PL using a series of fractionations by ethanol, hexane and acetone.

A patented process developed by Kearns et al. (1992) used acetonitrile for extracting PL from egg yolk sequenced by acetone washing or supercritical CO_2 (SC-CO_2) and chloroform-methanol washing for the separation of different PL based on their solubility in different solvents. In a recent study by Gadkowski et al. (2012), a purity of 100% of PL was obtained using fresh yolk dissolved in ethanol and later precipitated a few times with -20 ^{o}C acetone. The exact recovery was not reported; however, the total extraction yield of PL based on the initial yolk weight was 9.5%.

2.3.2. SC-CO_2 extraction

SC-CO_2, as an alternative technology to the conventional organic solvent extraction techniques, has been introduced as a sustainable and clean technology for lipid extraction and fractionation from different food matrices. It has been successfully applied at industrial scale for the separation of high value specialty oils.

When a substance is compressed and heated above its critical temperature and pressure, it has a liquid like-behaviour since it has a high density and at the same time it is like a gas due to its low viscosity. Therefore, the substance can have higher diffusivity due to its gas-like properties and strong solvent power due to its high density. Carbon dioxide is the most commonly used substance for the extraction of natural compounds and its phase diagram is presented in Figure 2.4. The critical point of CO_2 is at 7.4 MPa and 31.1 ^{o}C and it is a gas at ambient temperature and pressure; therefore, after extraction no residue stays in the extract following pressure reduction. Carbon dioxide is also a cheap and abundant gas in nature with no toxicity and flammability, and provides an oxygen-free environment during extraction, which makes it a great solvent for lipid extraction. However, due to its non-polar nature, CO_2 is not able to dissolve polar and slightly polar compounds such as PL. To overcome this problem, a polar co-solvent should be added to the system. For food and related applications, the co-solvent must be GRAS (generally regarded as safe). Ethanol and water or mixtures of both are the only co-solvents allowed for food applications. Although ethanol addition into the SC-CO_2 can result in the extraction of high value compounds that could not be extracted using neat CO_2, it diminishes the advantage of SC-CO_2 as a non-flammable solvent. In addition, ethanol should be removed from the final product, which adds further processing steps, increasing the cost (Temelli, 2009).

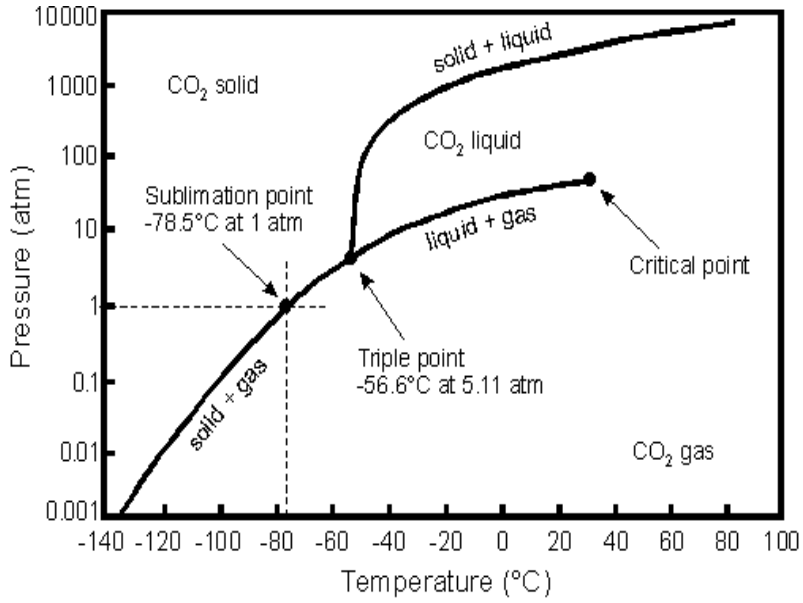

Figure 2.4. Carbon dioxide pressure-temperature phase diagram. (http://www.standnes.no/chemix/english/phase-diagram-co2.htm; Accessed on 29/12/2012).

The extraction of PL from food materials such as soybean, canola and egg yolk using ethanol addition as co-solvent has been studied by several researchers (Rossi et al., 1990; Temelli, 1992; Dunford and Temelli, 1995; Montanari et al., 1996; Dunford and Temelli, 1997; Boselli and Caboni, 2000; Shah et al., 2004; Aro et al., 2009). The insolubility of PL in CO_2 has been exploited as an advantage for PL fractionation through a two-step extraction technology. In the first step, neutral lipids are extracted using neat CO_2, leaving a fraction rich in proteins and polar lipids in the sample. In the second step, ethanol is added to the CO_2 stream as a co-solvent for PL extraction.

Dunford and Temelli (1995) could extract PL from canola through such a two-step extraction method using 10% ethanol in the second step, at 55.2 MPa and 70 ^{o}C. Not more than 200 ppm of PL could be detected in the extract if

ethanol concentration in SC- CO_2 was less than 8%. Their results showed that by increasing ethanol percentage, the amount of PL increased in the final extract. Montanari et al. (1996) also successfully extracted PL from soybean using such a two-step extraction protocol and 10% ethanol.

SC-CO_2 has been applied for cholesterol removal and PL extraction from egg yolk by several researchers. Froning et al. (1990) used several pressures and temperatures to extract cholesterol from dry yolk powder. By adjusting temperature and pressure, almost 2/3 of the available cholesterol in egg yolk was extracted. They found that by increasing temperature and pressure extraction yield increased. Boselli and Caboni (2000) could extract PL and neutral lipids at micro scale with one step extraction, without ethanol addition, using four steps of 30 min each for extraction at 51.7 MPa, 45 ^{o}C and 0.35 mL/min CO_2 flow rate. After each step, the system was depressurized and lipid extract was collected. However, later Shah et al. (2004) and Aro et al. (2009) could not extract PL in one step using neat CO_2. Aro et al. (2009) suggested the differences in the raw materials used such as moisture content and surface properties of dry yolk as well as the high pressure applied in the former study may be the reasons for co-extraction of PL together with neutral lipids using neat SC-CO_2.

Moisture content and particle size are critical factors impacting the extractability and recovery of lipids from biomaterials. SC-CO_2 performance on the extractability of lipids from liquid material or material with high moisture content such as fresh meat or yolk is inefficient and challenging. However, the presence of a certain amount of water can improve extractability of compounds

through shifting the selectivity of SC-CO_2 to certain compounds. For samples with very low moisture content, higher affinity of lipids to the matrix decreases the solubility of lipid in the solvent. In fact, for selective and efficient extraction of alkaloids such as caffeine and nicotine, pre-soaking of material in water is required (Zosel, 1981). However, excess amount of water in a sample can suppress the diffusivity of CO_2 through solubilisation of CO_2 in water, which in turn decreases the solubility of target material in the solvent. Also, carbonic acid produced by CO_2 solubilisation in water reduces pH, which affects the interaction between solvent and solute and extraction efficiency. It has been reported that the reduction of egg yolk moisture content from 50% to 30% and the addition of a co-solvent like ethanol or methanol could improve extractability of cholesterol (Zeidler et al., 1994). Froning et al. (1998) showed that water content of 7% could improve the extractability of neutral lipids and cholesterol from dry yolk. However, an additional increase in moisture content had a negative impact on the extractability of lipids.

Particle size of the material also plays an important role on extraction yield. It has been reported that lipids could be removed more efficiently from large particles in dry meat chunks and egg yolk (Froning et al., 1994, 1998). Lipid extraction from very small particles of egg yolk powder could be hindered due to the "channeling phenomenon" where the SC-CO_2 makes cave- or tunnel-like paths through the sample with very small particle size (King et al., 1989).

In PL extraction from egg yolk, Shah et al. (2004) extracted PC from dry egg yolk using the two-step extraction. The second step of extraction was

performed for 1 h at 41.1 MPa, CO_2 flow rate of 5 L/min (measured at 0.1 MPa and 25 ^oC) and 60 ^oC using 50% ethanol molar fraction in CO_2. The PC recovery was 49 g/kg of dried egg yolk but its purity was not reported. No PL could be extracted at 5-20% of ethanol addition. In a recent study by Aro et al. (2009), PL extraction from egg yolk was investigated using the anti-solvent property of CO_2. In an approach similar to Sim (1991), egg yolk lipids were extracted by ethanol and the ethanolic solution was stored at 4 ^oC overnight to separate the upper liquid phase rich in PL. The liquid phase was condensed to a certain volume and applied into a high pressure chamber through a spray nozzle set to 27 MPa, 70 ^oC and the SC-CO_2 flow rate of 0.25 L/min. The recovery of PC+PE was about 40-50% with a purity of PL at 99%. Using the two-step extraction technique, the recovery of PL was about 12-18% at 40 MPa, 70 ^oC and 15% ethanol. Despite the achievements made in PL extraction using SC-CO_2, the recovery of PL is low.

2.3.3. Biopolymer interactions as an approach for lipid separation

Polysaccharides are non-toxic, biocompatible and biodegradable polymers. Bioactive compounds separation using different polysaccharides from aqueous systems such as whey protein, milk and egg yolk has been investigated. Interactions between two biopolymers depending on the nature of biopolymers and applied conditions can be divided into four groups of interactions: electrostatic, steric exclusion, hydrophobic and hydrogen bonding (McClements, 2006). Other interactions are van der Waals interactions, which are weaker forces compared to the interactions listed above but can be important for self-association

of biopolymers such as proteins (Hoskin et al., 1998). The presence and magnitude of these interactions depend on biopolymer structure, molecular weight, charge density, the availability of functional groups and external factors such as pH, temperature, and ionic strength.

Phase separation phenomenon can be due to an associative or segregative type (Fig. 2.5). In associative separation, two biopolymers interact to form a complex which is not soluble in solution, and therefore, phase separation occurs. In segregative phase separation, mixing the two biopolymers is not thermodynamically favourable due to electrostatic repulsion or steric exclusion (Doublier et al., 2000). Modulation of external factors such as biopolymer concentration, pH and ionic strength may lead to specific type of products or a phase rich in a specific type of biopolymer. Several studies have been conducted on the types of interactions or possible separation of specific proteins from whey or milk proteins such as β-lactoglobulin and casein using polysaccharides like carrageenan or chitosan (Ausar et al., 2001; Casal et al., 2006; Lachkar et al., 2008). Cornwell and Kruger (1961) reported the precipitation of certain lipoproteins from blood serum at neutral pH using sulphated polysaccharides.

Adding polysaccharides to egg yolk has been specifically attractive for IgY isolation since some polysaccharides showed the ability of lipid precipitation from plasma to the granules. Hatta et al. (1988) used sodium alginate for primary precipitation of LDL during the IgY extraction process. Shah and Singh (1992) used carboxymethyl cellulose (CMC) for IgY separation from two-fold diluted egg yolk. Direct mixing of egg yolk with CMC solution (at final dilution of two

fold) caused co-precipitation of lipoproteins (lipid fraction) in granules. However, the addition of CMC to the plasma fraction formed a semi-solid phase and a subnatant liquid rich in IgY.

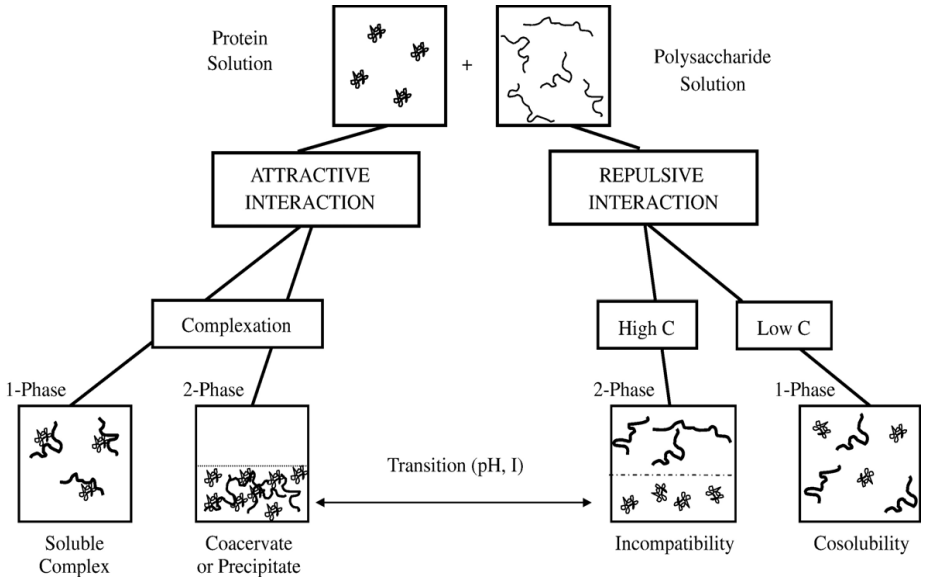

Figure 2.5. A schematic representation of possible structural rearrangement upon mixing two different biopolymers, Image was reprinted from McClements (2006) with permission.

Chang et al. (2000) studied the effects of sodium alginate, λ-carrageenan, CMC and pectin on the isolation of IgY from six-fold diluted egg yolk at pH 4-6. Pectin at pH 6 showed the best recovery of IgY (74%). They also studied the nature of interactions (ionic, hydrophobic or hydrogen bonding) through measuring the turbidity of yolk/polysaccharide complexes in the presence of different concentrations of sodium thiocyanate (NaSCN) or urea. It was

concluded that ionic bonds were major forces for lipoprotein-polysaccharide interactions.

Rojas et al. (2007) studied cholesterol removal from egg yolk using high methoxyl pectin. The best condition for cholesterol removal from egg yolk was at alkali pH 9 and about six-fold egg yolk dilution. It seemed that cholesterol co-precipitated with pectin to the pellet. Previous studies (Hatta et al. 1988, 1990; Shah and Singh, 1992) focused on IgY extraction where LDL usually co-precipitated with granules. LDL as an intact component has a routine application in the agriculture industry as a cryoprotective agent of animal semen (Moussa et al., 2002). Also, as already mentioned, LDL has a unique spherical nano-structure which may find applications in delivery systems. Separation of individual LDL particles has been performed through ultracentrifugation, which requires long hours and although the purity is high, the yield is very low. A method relying on egg yolk plasma separation, salting out precipitation and dialysis has been developed by Moussa et al. (2002) for LDL separation. LDL recovery with this technique is around 67%, however, it also requires long hours of dialysis.

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3. Physicochemical properties of leftover egg yolk after livetins removal[1]

3.1. Introduction

Egg yolk is an abundant and inexpensive source of proteins and phospholipids (PL) as described in previous chapters. Livetins, accounting for about 10% of fresh egg yolk protein composition, are water-soluble proteins with a heterogeneous structure consisting of α-, β- and γ-livetins at a ratio of 2:5:3 (Bernardi and Cook, 1960; Kovacs-Nolan and Mine, 2004, 2005). γ-Livetin (IgY), is analogous to mammalian antibodies (Mine, 2002). IgY from egg yolk is an important source as an immunotherapeutic agent with great potential for applications in oral vaccination and passive immunization against bacterial and viral pathogens (Mine, 2002).

Several techniques have been developed for IgY extraction (De Meulenaer and Huyghebaert, 2001), including a simple water dilution method described by Kwan et al. (1991). In this method, water-soluble proteins are separated in the supernatant after dilution of egg yolk, pH adjustment and centrifugation. Leftover egg yolk pellet after soluble proteins removal contains the majority of proteins and lipids in its emulsion structure. Therefore, it can be used as an emulsifier in the food industry. Kwan et al. (1991) showed that emulsions prepared from the leftover egg yolk had slightly higher emulsifying activity index and the mayonnaise prepared from the leftover egg yolk showed acceptable properties.

[1] A version of this chapter is to be submitted to LWT- Food Science and Technology for publication.

Egg yolk can be separated into two fractions; granules and plasma. Granules are insoluble at egg yolk natural pH. Plasma is the soluble phase, which contains LDL and livetins (Mcbee and Cotterill, 1979). Several groups studied factors affecting egg yolk and its fractions (granules and plasma) emulsifying properties under different enzymatic, thermal, ionic strength and pH conditions (Causeret et al., 1991; Aluko and Mine, 1997; Anton and Gandemer, 1997; Anton and Gandemer, 1999; Le Denmat et al., 1999; Mel'nikov, 2003; Kiosseoglou, 2004; Campbell et al., 2005; Nilsson et al., 2007; Sirvente et al., 2007; Daimer and Kulozik, 2008, 2009, 2010; Daimer and Kulozik, 2009; Buxmann et al., 2010 a,b). Results from some of these studies showed that depending on the environmental conditions, emulsifying properties of egg yolk proteins can change drastically. Granules exhibit good emulsifying properties, such as good adsorption on the oil interface and form emulsions with higher stability at higher pH or ionic strength conditions (Anton and Gandemer, 1997). At egg yolk's natural pH condition (pH 6), in a competitive adsorption among egg yolk proteins, LDL proteins are more surface active and can be adsorbed easily on the oil surface, while livetins mostly stay in the continuous phase (Nilsson et al., 2007).

Kwan et al. (1991) showed that by increasing dilution factor from 2 to 10 using distilled water at egg yolk natural pH 6, over 90% of egg yolk lipids precipitated in the granules, whereas the aqueous phase contained mostly soluble livetins with minor amount of lipids. The 10-fold dilution method is used commonly in IgY preparation from egg yolk. Livetins account for less than 2% of egg yolk dry matter, while the pellet obtained after IgY isolation is an abundant

source of phospholipids and proteins, which can be applied as an emulsifier or can be further processed for value added phospholipids extraction. Therefore, the objectives of this study were to determine the effect of soluble protein removal on the emulsifying and rheological characteristics of the leftover egg yolk pellet.

3.2. Materials and Methods

3.2.1. Egg yolk fractionation

White shell eggs (grade A), produced by Sparks egg farmers of Lucerne Inc. (Calgary, AB, Canada), were obtained from Safeway stores (Edmonton, AB, Canada). Egg yolks were separated from the albumen manually and rolled on Whatman paper to remove albumen from yolk. The vitelline membrane was punctured with a sharp blade and egg yolk content was collected in a beaker placed in an ice bath. Egg yolk pellet was prepared according to Kwan et al. (1991) with some modifications. The general scheme of the protocol is presented in Fig. 3.1.

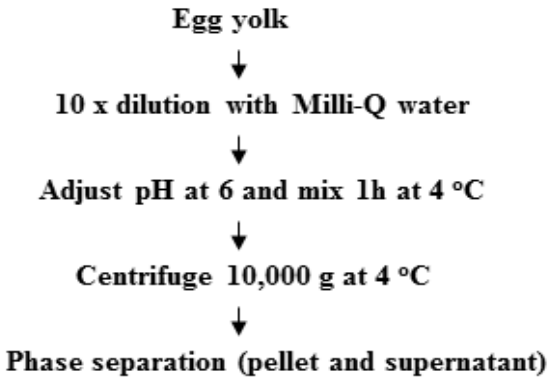

Figure 3.1. General scheme of egg yolk fractionation for IgY isolation and pellet formation.

Egg yolk was diluted 10-fold with MilliQ water and pH was adjusted to 6.0. using 1 N HCl. The yolk slurry was mixed for 1 h at 4 ^{o}C and was centrifuged at 10,000×g, at 4 ^{o}C for 15 min to separate water-soluble proteins. Then, the supernatant was separated from the pellet. The pellet was centrifuged once again under the same conditions and liquid was separated from the pellet. The egg yolk, pellet and supernatant were freeze dried (Labconco, model 7806020, Kansas, MO, USA) for proximate and protein profile analyses.

3.2.2. Preparation of reconstituted egg yolk

Supernatant, obtained from 10-fold dilution of egg yolk was divided into small containers and freeze dried. About 1.5-2 g of the freeze-dried supernatant was added back to about 20 g fresh pellet, containing about 43% dry matter, in order to replicate the composition of the original intact egg yolk (about 52% dry matter), which is referred to as reconstituted egg yolk. Emulsion properties of the reconstituted egg yolk were compared to those of the original yolk and pellet.

3.2.3. Characterisation of egg yolk fractions

3.2.3.1. Proximate analysis

Total lipid content was determined according to Hara and Radin (1978) using hexane and isopropanol as solvents and the lipid content was expressed as g/100g on dry weight basis. Total nitrogen content was determined by combustion method using a TruSpec CN analyser (Leco Corp., St Joseph, MI, USA). Total protein concentration was then calculated by multiplying the total nitrogen content by the conversion factor of 6.25.

3.2.3.2. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) for determination of protein profile

SDS-PAGE profiles of yolk and its fractions (pellet and supernatant) were performed using 10-20% Tris-HCl ready gels in a Mini-PROTEAN tetra cell (Bio-Rad Laboratories, Inc., Hercules, CA, USA) attached to a PowerPac Basic electrophoresis apparatus (Bio-Rad Laboratories Inc., Hercules, CA, USA). A high molecular weight protein marker was also obtained from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Before running the gels, lipids were removed from the freeze-dried samples by hexane/isopropanol (3/2, v/v), the lipid phase was separated by centrifugation at 10,000×g for 15 min and samples were then dried under a gentle stream of nitrogen. Samples were dissolved in 1X SDS-PAGE running buffer (Tris base 30%, Glycine 14% and sodium dodecyl sulphate 1%) at a concentration of 2.5 mg/mL and shaken in a thermomixer (Eppendorf, Hamburg, Germany) for at least 1 h at 20 ^{o}C to dissolve proteins. About 50 µL of protein solution was mixed with 45 µL Laemmli sample buffer and 5 µL of 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA) and heated at 90 ^{o}C for 5 min. About 20 µL of sample was loaded in a well and 1X SDS-PAGE running buffer was used. Gels were stained in a bath of staining solution, shaking gently on an orbital shaker (Lab-Line Max Rotator, Model 4631, Dobunq, IA, USA) for 1 h. The staining solution composition was 90 mL distilled water, 250 mL methanol, 50 mL acetic acid and 100 mL 1% Coomassie blue. After staining, samples were destained using destaining solution, composed of 500 mL methanol, 400 mL distilled water and 100 mL acetic acid. The gels were scanned using an

Alpha Innotech gel scanner (Alpha Innotech Corp., San Leandro, CA, USA) with FluorChem SP software.

3.2. 4. Emulsifying properties

3.2.4.1. Oil droplet size, flocculation index (FI) and coalescence index (CI)

The protein concentration was standardized at 30 mg/mL and pH was adjusted to pH 6, which is the natural pH of egg yolk. Oil-in-water emulsions were prepared by adding 20% (v/v) sunflower oil (Safeway-100% pure). Sunflower oil and protein sample were homogenized for 1.5 min at 20,000 rpm using a high-speed disperser (Ultra-Turrax T25; Janke & Kunkel IKA® Labortechnik, Staufen, Germany). Oil droplet size was measured by laser light diffraction (Mastersizer 2000S, Malvern Instruments Ltd., Chicago, IL, USA). The refractive index for water as dispersant was 1.33 and for oil was 1.46 at 20 ^{o}C. At least eight individual egg yolk, pellet and reconstituted yolk emulsions were prepared for the characterisation of emulsions. Flocculation and coalescence indices were calculated according to Sirvente et al. (2007). Oil droplet size of fresh samples was measured after sample dilution with 1% (w/v) SDS. For flocculation determination, emulsions were kept for 4 h at ambient temperature (20 ^{o}C). After 4 h, 1 mL of emulsion was mixed with MilliQ water. Flocculation index was calculated as the ratio of volume weighted mean of oil droplet size (*d4,3*) measured after 4 h in water without 1% SDS addition and that of original fresh oil droplet as follows:

Flocculation index = (*d4,3* of flocs/*d4,3* of droplets) × 10

For coalescence index determination, prepared emulsions were stored stationary at 5 ^{o}C for 7 days. After 7 days, coalescence index was calculated by estimating the specific surface area (m^2/mL, as SS=6/*d3,2*), where *d3,2* is the surface weighted mean diameter of the oil droplet. Before coalescence index measurement, samples were diluted 10-fold in 1% SDS. The coalescence index was calculated as follows:

Coalescence index= $(SS_0\text{-}SS_7/SS_0)\times100$, where SS_0 and SS_7 are the specific surface areas of oil droplets of the emulsions stored for 0 and 7 days, respectively.

3.2.4.2. Zeta potential (ζ)

Surface charge of oil droplets of emulsions prepared from yolk and pellet was measured using Zeta sizer 2000 (Malvern Instruments, Worcs, UK). About 0.5 mL of emulsion was diluted to 100 mL using MilliQ water while keeping pH constant at pH 6 by adding 0.1 M HCl or NaOH. At least eight emulsions from egg yolk and pellet were prepared for zeta potential measurement. Each measurement was based on an average of five readings at 20 ^{o}C.

3.2.4.3. Surface hydrophobicity

For the surface hydrophobicity test, 1-anilino-naphthalene-8-sulfonate (ANS) assay was performed using a spectrofluorophotometer (Shimadzu, model RF-5301PC, Kyoto, Japan) according to Rao et al. (1978) and Yan et al. (2010). Different dilutions of emulsions from 0 to 0.12% were prepared in 0.01 M phosphate buffer at pH 7. Fifteen μL of 8 mM ANS was added to 30 mL of sample and incubated for 15 min in the dark. Fluorescence intensity was measured

at excitation and emission wavelengths of 390 and 470 nm, respectively. Emission slit width was set at 5 nm. The difference in fluorescence intensity between (buffer+ANS) and (buffer+sample+ANS) was plotted against emulsion concentrations and the slope of the regression line was reported as surface hydrophobicity, for which three individual samples of pellet and yolk emulsions were prepared and measured.

3.2.5. Unadsorbed protein profile of emulsions from intact egg yolk

Unadsorbed protein profiles of intact egg yolk emulsions were examined using SDS-PAGE. Egg yolk emulsions were kept stationary at ambient temperature for about 4 h for the flocculation study. The aqueous subnatant phase was separated from the emulsion and then freeze dried. Samples for SDS-PAGE were prepared according to the method described under Section 3.2.3.2.

3.2.6. Rheological measurements

Rheological properties of egg yolk and fresh pellet were determined using a controlled stress rheometer (PAAR Physica UDS 200, Glenn Allen, VA, USA) equipped with a peltier heating system. Parallel plate geometry (MP30, 25 mm diameter) with a gap of 0.5 mm was employed at 20 ^{o}C. Samples were rested for at least 15 min prior to measurements, allowing them to release the stress during sample loading. Silicon oil of low viscosity (5 mPa.s) was applied to avoid sample drying during analysis. Strain sweep tests were performed from 1 to 100% strain at a frequency of 1 Hz. Apparent viscosities of egg yolk and its pellet were also determined by increasing the shear rate from 3 to 100 s^{-1}. Time sweep was

performed at 0.5 Hz frequency and 3% strain, within the linear viscoelastic region.

3.2.7. Statistical analysis

Statistical analysis was performed by one-way analysis of variance using GRAPHPAD PRISM (Graph pad software, San Diego, CA, USA) at a confidence interval of 95% ($p < 0.05$). Means were compared to determine significant differences at $p < 0.05$ by using the Tukey's HSD test.

3.3. Results and discussion

3.3.1. Proximate composition of egg yolk and pellet

From 100 g of yolk, 104 ± 13 g (n=5) of pellet was obtained after 10-fold water dilution and centrifugation. Table 3.1 shows the proximate composition of egg yolk and pellet.

Table 3.1. Moisture, lipid and protein contents (%) of fresh yolk and leftover egg yolk.

	Moisture (%)	**Protein (%)**	**Lipid (%)**
Yolk (n=5)	48±0.7	16.5±1.2	33.8±0.5
Pellet (n=5)	57±0.3	12.7±0.9	28.8±1.4

Compared to egg yolk, the moisture content of pellet increased from 48% to 57%, while its lipid and protein contents decreased from 33.8% and 16.5% to 28.8% and 12.7%, respectively. In this study, 10-fold water dilution and centrifugation of egg yolk resulted in an opaque supernatant, indicating the presence of dispersed components such as proteins. According to Kwan et al. (1991), after increasing the dilution factor from 2 to 10-fold at pH 6.0 over 90%

of the lipids in the egg yolk was precipitated in the pellet. Results of this study were in agreement with the previous report.

3.3.2. Protein profile of egg yolk fractions

SDS-PAGE profiles of egg yolk, pellet and supernatant were studied to identify protein types transferred to the supernatant after 10-fold water dilution and centrifugation. The protein profiles of egg yolk and its separated fractions (pellet and supernatant) were presented in Fig. 3.2.

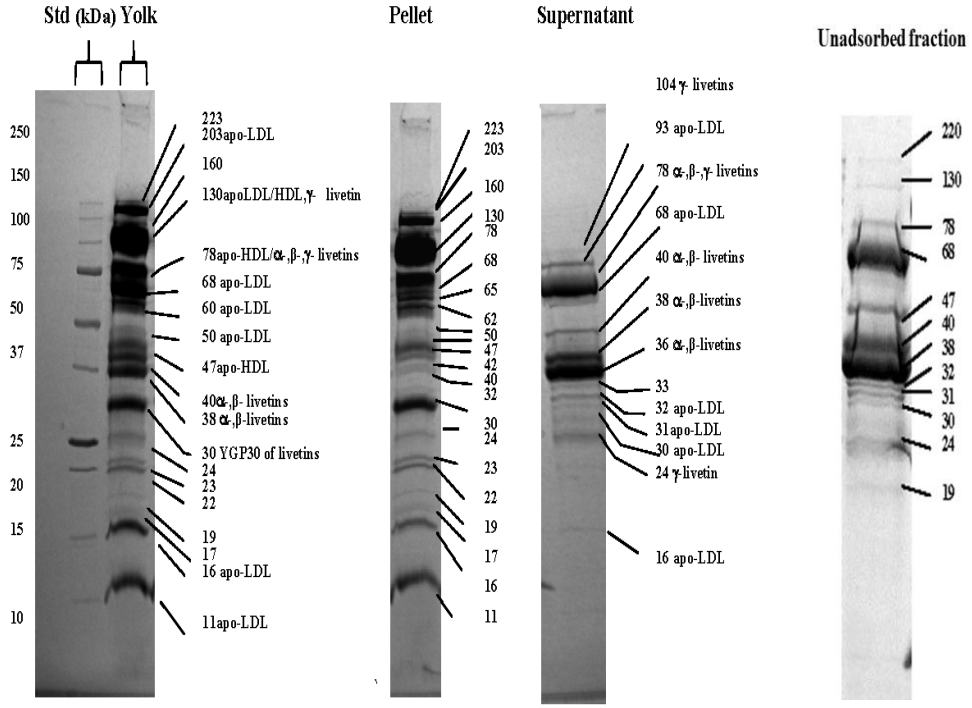

Figure 3.2. SDS-PAGE profile of egg yolk and its fractions (pellet and plasma) and the protein, which did not adsorb to oil droplet surface of egg yolk emulsions (unadsorbed fraction).

Overall, at least 25 protein bands were detected in intact yolk, pellet and supernatant fractions in this study. Fig. 3.2 showed 19, 22 and 14 bands in yolk, pellet and supernatant fractions, respectively. Proteins with close molecular weights overlap in SDS-PAGE gel, which makes their identification difficult; however, removal of soluble proteins allowed some overlapped bands in egg yolk to be detected in the pellet; therefore, it was possible to observe more bands in the pellet.

Table 3.2 summarizes the names and molecular weights of the different proteins observed yolk, pellet and supernatant. Three proteins with molecular weights of 160, 42 and 33 kDa were detected in SDS-PAGE profiles of yolk, pellet and supernatant, but no reference could be found in the literature to relate them to any of the major egg yolk proteins. Bands with molecular weights of 104, 78, 40, 38, 36, 24 found in the supernatant phase were from livetins. Some apoproteins from LDL (93, 68, 32, 31, 30, and 16 kDa) also remained in the supernatant. Protein band of 65 kDa seems to belong to IgY, which remained in the pellet after yolk dilution and did not migrate to the supernatant. Overall, SDS-PAGE profile showed that soluble proteins (livetins) and some LDL remained in the supernatant after 10-fold dilution of yolk. The band of 130 kDa was the major band with the highest density in both yolk and pellet fractions but not in the supernatant. Dauphas et al. (2006) reported a major apoprotein from LDL with a molecular weight of 130 kDa in egg yolk, while protein bands with molecular weights of 122, 93, 85 kDa were also reported as LDL apoproteins (Guilmineau et al., 2005).

Table 3.2. Proteins and their related molecular weights for intact egg yolk, leftover pellet and the supernatant.

MW (kDa)	Fraction	Related protein	Reference
223	yolk/pellet	Apo-LDL	(Mine, 1998)
203	yolk/pellet	Apo-LDL	(Guilmineau et al. 2005)
160	yolk/pellet	N/A	
130	yolk/pellet	apo-LDL/HDL,γ-livetin	(Guilmineau et al, 2005)
104	supernatant	γ-livetin	(Guilmineau et al. 2005; Jolivet etal. 2008)
93	supernatant	Apo-LDL	(Guilminea et al. 2005)
78	yolk/pellet/supernatant	Apo-HDL/α-,β-,γ-livetins	(Guilmineau et al. 2005)
68	yolk/pellet	Apo-LDL	(Guilmineau et al. 200)
65	pellet	Apo-LDL, γ- livetin	(Guilmineau et al. 2005; Jolivet et al. 2008)
62	pellet	Apo-LDL	(Guilmineau et al. 2005)
60	yolk	Apo-LDL	(Guilmineau et al. 2005)
50	yolk/ pellet	Apo-LDL	(Nilsson et al.2007)
47	yolk/pellet	Apo-HDL	(Guilmineau et al. 2005)
42	pellet	N/A	(Guilmineau et al. 2005)
40	yolk/pellet/supernatant	α-,β- livetins	(Guilmineau et al. 2005)
38	yolk/supernatant	α-,β-livetins	(Guilmineau et al. 2005)
36	supernatant	α-,β-livetins	(Guilmineau et al. 2005)
33	supernatant	N/A	
32	Pellet/supernatant	Apo-LDL	(Nilsson et al. 2007)
31	Pellet/supernatant	Apo-LDL	(Nilsson et al. 2007)
30	yolk/pellet/supernatant	HDL, YGP30 of livetins	(Jolivet et al. 2008)
24	yolk/pellet/supernatant	γ-livetin	(Guilmineau et al. 2005; Jolivet et al. 2008)
23	yolk/pellet	Apo-LDL	(Guilmineau et al. 2005)
22	yolk/pellet	Apo-LDL	(Guilmineau et al. 2005)
19	yolk/pellet	Apo-LDL	(Guilmineau et al. 2005)
17	yolk/pellet	Apo-LDL	(Guilmineau et al. 2005)
16	yolk/pellet/supernatant	Apo-LDL	(Guilmineau et al. 2005)
11	yolk/pellet	Apo-LDL	(Guilmineau et al. 2005)

HDL has been reported to contain apoproteins with molecular weights of 110, 100, 80, 50 and 35 kDa (Anton, 2007). Therefore, the 130 kDa band in the yolk obtained in this study might be related to apoproteins from LDL as well as, containing some other types of proteins such as apo-proteins from HDL and

livetins. IgY has been identified as having three bands with molecular weights of 104, 74 and 24 kDa according to Guilmineau et al. (2005).

Guilmineau et al. (2005) also reported 12 bands with molecular weights of 221, 203, 122, 93, 85, 68, 62, 55, 21, 20, 17, 5 kDa as LDL apoproteins and 110, 78, 47, 31 kDa as HDL apoproteins. In this study, bands with 223, 203, 130, 93, 68, 65, 62, 60, 50, 32, 31, 23, 22, 19, 17, 16 and 11 kDa, found in the yolk, pellet and supernatant fractions, were related to LDL apoproteins. Band with a molecular weight of 203 may come from apoproteins from LDL, IgY and α-livetin.

3.3.3. Emulsion properties

Emulsion properties of egg yolk and its pellet as well as its reconstituted yolk were evaluated in terms of oil droplet size and flocculation and coalescence indices as shown in Table 3.3. There was no significant difference ($p<0.05$) between the yolk, pellet and reconstituted yolk in terms of oil droplet size and flocculation index (Table 3.3). However, the coalescence index of emulsions prepared with the pellet was significantly ($p<0.05$) higher than those of yolk and reconstituted egg yolk emulsions. Generally, larger oil droplet size is correlated with lower emulsifying stability and higher probability of coalescence incidence in emulsions.

Flocculation and coalescence indices are measures of emulsion stability. In flocculation, oil droplets aggregate together but they do not merge to form a larger, single oil droplet (McClements, 2005). Coalescence is regarded as a severe level of emulsion instability in which interfacial films of oil droplets are ruptured

and they merge together to form bigger oil droplets (McClements, 2005). Emulsions prepared with the pellet had substantially higher coalescence index compared to emulsions prepared with the yolk. It seems that the removal of soluble proteins induced instability in emulsions prepared from leftover pellet through the coalescence mechanism.

Table 3.3. Emulsion properties (n=8) of yolk, pellet and reconstituted yolk in terms of oil droplet size, flocculation index (FI) and coalescence index (CI).

Sample	Oil droplet size (µm)	FI (%)	CI (%)
Yolk	18±1[a]	10±2[a]	1±1 [a]
Pellet	16 ±0.6[a]	11±2[a]	8 ±3 [b]
Reconstituted yolk	18±1[a]	10±1 [a]	2±2[a]

[a,b]Values within the same column followed by a different letter are significantly different (p<0.05).

All egg yolk proteins have emulsifying activity. LDL apoproteins are considered to be the main contributors to egg yolk emulsifying properties (Aluko and Mine, 1998; Le Denmat et al., 2000; Anton et al., 2003). Although livetins can be adsorbed on an oil droplet surface to form emulsions (Le Denmat et al., 2000; Nilsson et al., 2007), in a competitive system like whole egg yolk, containing several proteins, livetins were not adsorbed on an oil droplet surface. Therefore, it was concluded that livetins have less impact on egg yolk emulsifying activity. Anton and Gandemer (1997) thought apoproteins have higher flexibility and hydrophobicity in their structure, which enables them to be adsorbed faster and stay longer on an oil interface. On the other hand, livetins are globular proteins and possess a rigid structure, which makes their adsorption on the oil

droplet surface quite difficult (Kiosseoglou and Sherman, 1983; Aluko and Mine, 1998).

In order to understand which proteins from intact yolk were not adsorbed on oil droplet surface and whether the protein profile of the unabsorbed fraction is similar to that of the supernatant separated from egg yolk after 10-fold dilution, egg yolk emulsions were kept stationary for 4 h at ambient temperature, the separated subnatant was collected and its protein profile was examined using SDS-PAGE. As Fig. 3.2 (unadsorbed fraction) indicates, about 12 distinct protein bands with molecular weights of 220, 130, 78, 68, 45, 40, 38, 32, 31, 30, 24 and 19 kDa were detected in SDS-PAGE gel, representing proteins which could not be adsorbed onto the oil droplet surface and therefore remained in the aqueous phase. Bands with molecular weights of 240, 68, 32, 31 and 19 kDa were protein fractions from LDL and bands with molecular weight of 40, 38, 30 and 24 were from livetins. Bands of 130 and 78 kDa could be unadsorbed proteins from LDL or livetins. A band with 45 kDa molecular weight was probably from livetins (Guilmineau et al., 2005)

These results are in good agreement with previous findings of Anton et al. (2003) and Nilsson et al. (2007). They reported that in a competitive adsorption among egg yolk proteins, some apoproteins and livetins from egg yolk cannot be adsorbed onto the oil droplet surface during emulsification. Some proteins identified in the supernatant, i.e. protein bands with 68, 32, 31 kDa from LDL, 40 kDa from α, β-livetins and 24 kDa from γ-livetins were among the proteins unabsorbed on the oil droplet surface.

Despite the fact that some apoproteins and livetins, which were also found in the supernatant that could not be adsorbed onto the oil droplet surface in egg yolk emulsions, the leftover pellet showed lower stability in terms of coalescence. Hydrophobicity tests showed that soluble proteins removal increased the surface hydrophobicity of the pellet emulsions (12,820±47), compared to that of egg yolk (10,750±92). On the other hand, zeta potentials of the emulsions prepared with yolk and pellet were similar (-10.68±2.2 vs. -10.95±1.3 mV) and could not answer questions regarding the stability of yolk and pellet emulsions. The method employed for hydrophobicity analysis was originally developed for the measurement of the surface hydrophobicity of proteins (Rao et al., 1978; Yan et al., 2010). However, in this study, the surface hydrophobicity of emulsion droplets prepared from egg yolk and the pellet were evaluated in order to assess if there was any correlation between the coalescence index and hydrophobicity of the emulsion system. Considering the fact that over 40% of LDL apoproteins are hydrophobic (Dauphas et al., 2006), at the same protein concentration, pellet may contain more hydrophobic proteins. While proteins with more hydrophobic side chains can be adsorbed faster on the hydrophobic oil surface, they may also have more hydrophobic groups exposed to the continuous phase. Higher surface hydrophobicity of oil droplets may induce stronger hydrophobic interaction among oil droplets, which in turn might explain the higher coalescence instability of the emulsions prepared from the pellet.

It should be noted that the protein profile results indicated the existence of LDL apoproteins in the supernatant (aqueous) phase. Therefore, higher instability

of emulsions prepared from the pellet could not only be related to the absence of livetins but it might also be affected by the loss of some apoproteins and lipids. According to Mine (1998), PL and even cholesterol from LDL positively affect egg yolk adsorption on the oil interface, increasing yolk emulsion stability. Results presented in Table 3.3 imply that soluble proteins removal in fact did not alter emulsifying activity of egg yolk but it significantly affected emulsion stability in the term of coalescence.

To further demonstrate the importance of soluble proteins for emulsion stability, freeze-dried supernatant fraction obtained from egg yolk dilution was added back to the leftover pellet to return the pellet to its original yolk composition. Emulsion properties of the reconstituted egg yolk were also shown in Table 3.3. Coalescence index of reconstituted egg yolk was similar to that of the original egg yolk (2 vs. 1), and interestingly soluble proteins addition into the pellet significantly ($p<0.05$) decreased coalescence index compared to that of the emulsions made from the pellet (2 vs. 8). This confirmed that water-soluble fraction obtained after 10-fold dilution played a significant role in the stability of yolk emulsion. Emulsifying activity generally refers to the ability of protein or surfactant to migrate, unfold and form a film around oil droplets; while emulsion stability is related to the resistance against flocculation and coalescence during storage as discussed above. A good balance of hydrophilic and hydrophobic chains and the viscoelastic properties of proteins on the oil droplet surface are some of the key factors that define an emulsion system's stability against coalescence. In addition to the interfacial properties of proteins, physical

properties such as viscosity are also important for the stability of an emulsion system.

Proteins on the interfacial film have two main functions: a) to decrease interfacial tension, and b) to form a viscoelastic barrier to support the oil droplet and to prevent its disruption. In this study, it was demonstrated that some livetins and some apoproteins could not be adsorbed onto the oil droplet surface. However, unadsorbed proteins are soluble and their presence in the continuous phase of the emulsion may increase the viscosity of the continuous phase and in turn slow down oil droplet movement towards each other. This mechanism may also explain why the addition of soluble proteins into the pellet to form the reconstituted yolk improved stability against coalescence.

3.3.4. Rheological properties of intact egg yolk and pellet

3.3.4.1. Strain sweep

Changes in yolk structure and its flow behaviour after soluble proteins removal may affect its technological aspects during processing and final product applications. Visual observation of the egg yolk and pellet samples indicated changes in the yolk structure and its fluidity after soluble proteins removal. Egg yolk had a more fluid character, but the pellet had a creamy and paste-like structure, which was resistant to flow. However, according to the strain sweep results (Fig. 3.3), both pellet and yolk exhibited liquid-like behaviour in the linear viscoelastic range, as viscous behaviour dominated the elastic one (G”> G’).

Strain sweep results showed that the pellet had higher G’ and G” values compared to those of egg yolk, indicating that the pellet has a stronger structure

with more resistance to applied stress. Loss factor (tan δ= G”/G’), a parameter showing the ratio of viscous and elastic portions of a viscoelastic material, was lower for the pellet. When G’ and G” are balanced (G’=G”), it means that the material behaves in a borderline between a liquid and a solid “gel” like structure. In this study, G’ and G” values for the pellet became closer i.e. tan δ was smaller for the pellet.

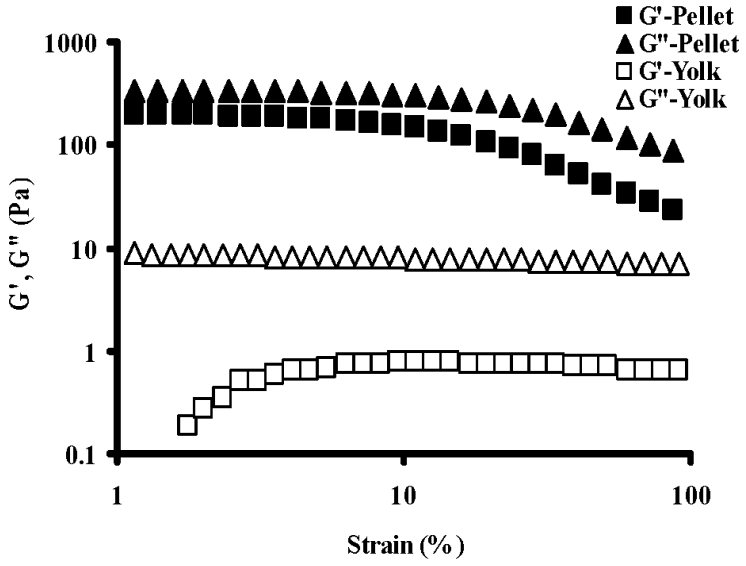

Figure 3.3. Strain sweep of yolk and pellet, G': Storage modulus (Pa), G": Loss modulus (Pa).

As shown in Table 3.1, the pellet contained less solid material as some proteins and lipids were replaced with water in the pellet structure. Nevertheless, both primary viscous and elastic moduli were higher for the pellet; therefore, the increase in G’ and G” cannot be related to the presence of more concentrated solid material in the pellet macrostructure. Up to 15% strain, G’ and G” for the pellet

were constant, indicating the limit of the linear viscoelastic region and above that strain, G' and G'' values decreased (Fig. 3.3). It means some interactions between the components in the pellet structure started to break at higher strain levels. The drop in the G' and G'' was not evident for the yolk, considering its more liquid behaviour.

It is apparent that the interactions between components play an important role and dictate the viscoelastic behaviour. Two hypotheses may be suggested for the higher viscoelastic moduli values of the pellet: a) the increase in water content may increase the chance for hydrogen bond formation between some amine side groups of proteins and water, b) the change in the ratio of proteins may impact the extent of different types of interactions between proteins. As mentioned above, livetins are less hydrophobic compared to LDL apoproteins (Jolivet et al., 2008). Therefore, separation of livetins and more soluble proteins from egg yolk may have increased the level of hydrophobic interactions, which was demonstrated earlier with the higher S_o value of emulsions from pellet (Table 3.3), resulting in higher G' and G" values.

3.3.4.2. Viscosity

As expected, the viscosity of pellet was higher than that of yolk (Fig. 3.4). Pellet clearly showed a shear-thinning behaviour (Fig. 3.4), while egg yolk seemed to have a constant viscosity with increasing shear rate.

According to Ibarz and Sintes (1989) as well as Telis-Romero et al. (2006), egg yolks with total solid contents of 43.3% and 46% showed slight pseudoplastic behaviour with a flow behaviour index of n=0.87 and n=0.857,

respectively, at 20 ^{o}C. In this study, yolk with an average moisture content of 48% (or solids content of 53%) exhibited a Newtonian behaviour with a flow behaviour index of n=1.02; however, the leftover pellet clearly had a shear-thinning behaviour with a flow behaviour index of n=0.33.

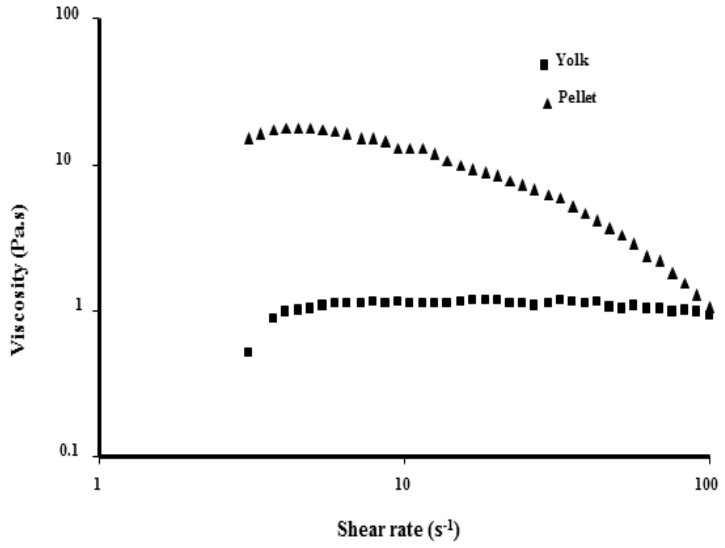

Figure 3.4. Viscosity of egg yolk and leftover pellet at shear rates of 3-100 s^{-1}.

3.3.4.3. Time sweep

A time sweep test can give information about the changes in the viscoelastic behaviour of materials i.e. their structural strength over time. Structural strength may remain unchanged, increase or decrease over time. An increase in structural strength may occur for polymers due to increasing number of molecular entanglements in dispersions, and gelation due to interactions such as hydrogen bonding. Results of time sweep test for yolk and pellet are shown in Figure 3.5.

G’ and G” curves for egg yolk crossed after 300 min (5 h), while for the pellet this happened after 440 min (over 7 h). Such a crossover indicates the formation of a network due to the interactions between proteins and changes from a liquid-like viscous behaviour (G’<G”) to a solid-like elastic behaviour (G’>G”). The partial removal of soluble proteins from egg yolk and their replacement with water resulted in a more liquid-like system pellet.

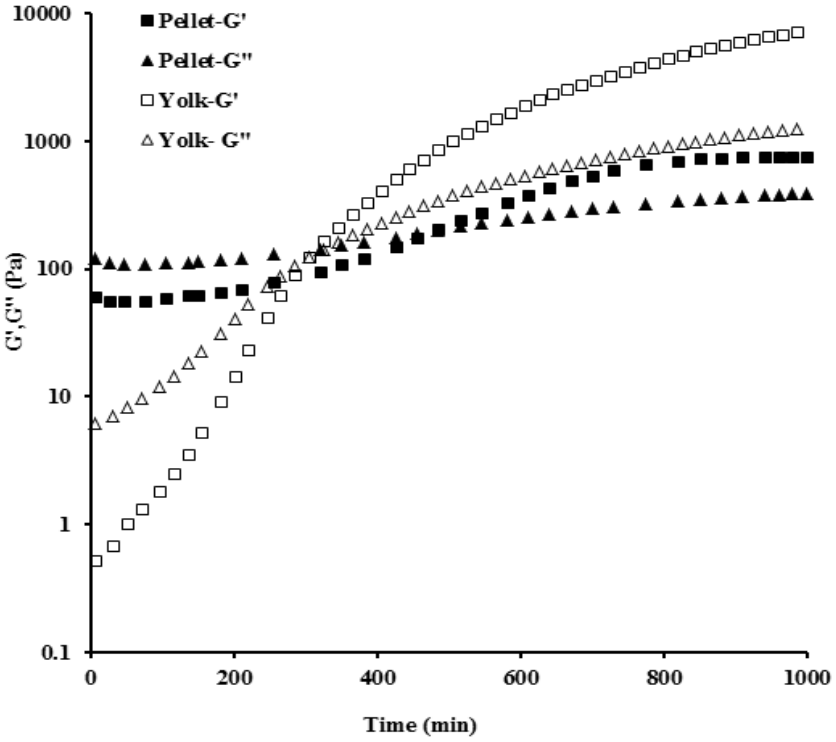

Figure 3.5. Time sweep of yolk and pellet, G': Storage modulus (Pa), G": Loss modulus (Pa).

Therefore, lower protein content in the pellet (Table 3.1) due to dilution together with its primary higher viscosity is probably responsible for the observed delay in network formation with the pellet over time. In other words, the higher

chance for entanglement and possible physical interactions between egg yolk components increase the chance for bonding and network formation in a shorter period of time. After the cross-over point, continuing the time sweep test for up to 1000 min showed a steady increase in G' and approaching a plateau for egg yolk, whereas a plateau was reached for the pellet. This indicates that a stable network structure was established. Both G’ and G” values were higher for the yolk after the crossover point, indicating higher entanglement and physical interactions between egg yolk components due to its higher solid content.

The changes in the structure of egg yolk after soluble proteins removal may be important from processing aspects. Overall the pellet showed resistance to flow and higher viscosity at lower shear rates probably due to some hydrogen or hydrophobic bonding; however, the viscosity decreased at higher shear rates. This type of rheological behaviour must be considered as one of the processing factors, which may affect final product properties.

3.5. Conclusions

Egg yolk dilution and soluble protein removal from egg yolk for IgY extraction leaves an abundant amount of pellet as a leftover product. Physical and chemical tests showed that soluble proteins removal had a substantial effect on egg yolk physicochemical properties. Emulsion prepared using the pellet was less stable compared to egg yolk, as demonstrated by a higher coalescence index. Egg yolk rheological properties after soluble proteins removal changed in terms of its viscoelastic behaviour. Pellet showed a higher viscosity and required a longer time to form a network. Findings of this study would be beneficial for the egg

yolk processing industry in terms of technological aspects and demulsification approaches for the recovery of high value components from leftover egg yolk pellet.

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4. Effect of enzymatic treatments on physicochemical properties of leftover egg yolk pellet after IgY separation[2]

4.1. Introduction

About 80% of egg yolk phospholipids (PL) are choline derivatives (Palacios and Wang, 2005a). Choline, an essential nutrient for brain and neuron systems development, is found abundantly in egg yolk phosphatidylcholine (PC). Choline-rich PL of egg yolk are routinely added to the formulations of infant formula and other pharmaceutical products; therefore, proteins and PL of egg yolk have broad applications in nutraceutical and pharmaceutical sectors (Zeisel, 1992). Leftover egg yolk after IgY extraction through 10-fold dilution contains majority of lipids (90%) and can be a good source for high-value PL extraction.

Egg yolk PL are extracted mostly by applying organic solvents such as hexane for deoiling of dried egg yolk, followed by PL extraction from deoiled material using ethanol (Palacios and Wang, 2005b; Gadkowski et al., 2012). In addition to a significant deterioration in PL quality, technologies involving extensive use of solvents have become increasingly restricted due to the environmental and safety concerns. Supercritical CO_2 (SC-CO_2) extraction had been applied as such an alternative technology for PL recovery from dried egg yolk but with limited success due to the low recovery (Aro et al., 2009).

All lipids in egg yolk, including PL are complexed with apoproteins to form lipoproteins through non-covalent bonds (Burley and Vadehra, 1989). The

[2] A version of this chapter is to be submitted to the Journal of the American Oil Chemists' Society for publication.

presence of non-covalent bonds between PL and apoproteins in the LDL structure and a stable emulsion system may hamper the extraction of PL from egg yolk using SC-CO_2 as an alternative extraction technology. Therefore, an alternative technique, targeting minimal use of organic solvents for PL extraction, requires destruction of the emulsion structure of the egg yolk. Enzyme-assisted demulsification has been investigated as an approach for oil recovery from cream formed during aqueous extraction of oil from oilseeds (Wu et al., 2009; Towa et al., 2010). Enzymatic treatments were also applied to egg yolk but mainly to improve its emulsion properties and heat stability during processing (Daimer and Kulozik, 2008; Buxmann et al., 2010).

This study focused on the modification of egg yolk functional properties, after soluble proteins removal, hereafter called pellet, through enzymatic hydrolyses. Unlike the previous studies, which mainly focused on improving the emulsion stability of egg yolk through enzymatic modification, the target of the current study was to destabilise the pellet using enzymatic treatments. Enzymatic treatment of egg yolk may de-stabilise the emulsion and weaken the interactions between PL and apoproteins thus facilitate PL recovery in subsequent stages. Therefore, the specific objectives were to investigate the effect of simultaneous proteolytic and lipolytic hydrolyses on degradation of major pellet constituents such as lipoproteins on the emulsion properties of the pellet in terms of oil droplet size and coalescence instability, phase separation behaviour, cream content, and PL recovery in the final creams obtained after phase separation. Findings from

this study may find applications in developing eco-friendly techniques for PL extraction from egg yolk.

4.2. Materials and Methods

4.2.1. Egg yolk pellet preparation

Egg yolk fractionation and pellet preparation were performed as described in Section 3.2.1. A general scheme of the overall experimental protocols employed in this study, described in more detail in the following sections given in Figure 4.1.

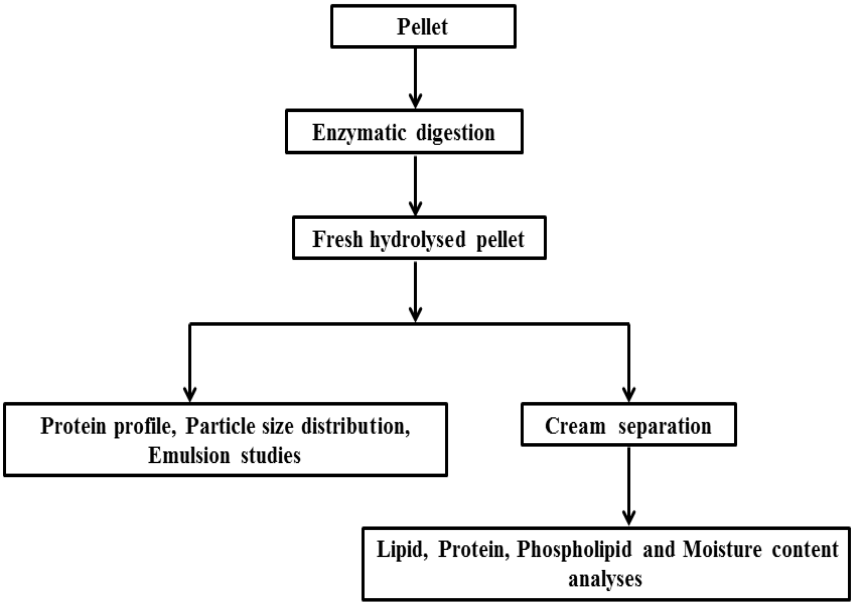

Figure 4.1. Schematic summary of experimental design.

4.2.2. Enzymatic treatment

For enzymatic treatments, a suspension was prepared first by mixing the pellet with MilliQ water to obtain a concentration of 7% (w/v) of pellet dry

matter, targeting a protein concentration of 20 mg/mL in the final suspension. The pellet suspension was mixed at 4 ^{o}C for 2 h using a magnetic stirrer to obtain a homogenous slurry, and then hydrolysed by a combination of Lipase AY30 (from *Candida rugosa*, a powder triacylglycerol lipase, active on short, medium and long fatty acid chains on *sn*-1, 2 and 3 positions of triacylgylcerol, Amano Enzyme Inc, Elgin, IL, USA) with one of the following six proteases: Protease A (from *Aspergillus oryzae*, a powder enzyme complex of proteinases and peptidases, minimum proteinase activity 20,000 units/g) at pH 6 and 50 ^{o}C, Acid Protease II (from *Rhizous niveus*, a powder enzyme complex of proteinases and peptidases, minimum proteinase activity 10,000 units/g) at pH 5 and 45 ^{o}C, Protease P (from *Aspergiluus melleus*, a powder enzyme complex of proteinases and peptidases, minimum proteinase activity 60,000 units/g) at pH 6 and 40 ^{o}C, Protease M (from *Aspergillus oryzae*, a powder acid proteolytic enzyme, minimum proteinase activity 5,500 units/g) at pH 5 and 50 ^{o}C , all kindly provided by Amano Enzyme, Inc. (Elgin, IL, USA), and Protex 51FP (from *Aspergillus oryzae*, a powder endo/exo-peptidase complex, minimum activity 400,000 HU/g)) at pH 7 and 50 ^{o}C and Protex 7L (from *Bacillus amyloliquefaciens*, a liquid endopeptidase 1,600 AU/g) at pH 6.5 and 45 ^{o}C, gifted by Genencor International Inc. (Rochester, NY, USA). Both protease and lipase were added at a level of 2% (w/w or v/w), based on the pellet dry matter weight.

The above enzymes were selected based on the phase separation behaviour of the pellet hydrolysates after enzymatic treatment during extensive preliminary

investigation of a large number of enzymes. No further analyses were performed to determine the activity level of the selected enzymes and the temperature and pH conditions were selected considering the optimum activity range provided by the manufacturers. The concentration level of enzymes was selected based on preliminary tests, taking into account the typical concentration ranges reported in the literature for such enzymes. Enzyme concentration was kept constant for comparison purposes.

Hydrolysates were centrifuged at 5 ^{o}C, 6,000×g for 30 min, and phase separation behaviour was observed. The pellet hydrolysates showing a triphasic system, including a cream phase were selected for further studies. However, a considerable amount of lipid was distributed in the middle liquid and pellet fractions. Considering the fact that lipids in egg yolk are all associated with the apoproteins of lipoproteins, it was hypothesized that including a lipase, which would hydrolyse the triglyceride core of lipoproteins may facilitate the access of proteolytic enzymes to proteins and result in efficient "demulsification" of the pellet. Therefore, Lipase AY30 a "triacylglycerol lipase," which can hydrolyse short, medium and long fatty acid chains from *sn*-1,2 and 3 positions of triglycerols was added in combination with the different proteases for enzymatic hydrolysis.

Egg yolk hydrolysis with Lipase AY30 alone in the absence of proteolytic enzymes did not result in the formation of a distinct cream phase. A distinct cream phase, rich in lipids, obtained after the use of a combination of selected

proteolytic enzymes with Lipase AY30 could be easily separated from the subnatant aqueous phase in a separatory funnel or brief centrifugation.

Hydrolysis was performed in a jacketed vessel to maintain temperature, while stirring at 300 rpm using a magnetic stirrer. The pH of slurry was maintained constant using 0.5 M NaOH or HCl dispensed by a Titrator (Metrohm, Buckingham, UK). After 3 h, hydrolysis was terminated by increasing the temperature to 90 ^{o}C and holding the samples for 10 min to inactivate the enzymes. Then, hydrolysates were immediately chilled by placing them in an ice bath. Later, hydrolysates were transferred to a pre-weighed beaker and their final weight was determined.

4.2.3. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Samples were freeze dried prior to SDS-PAGE analyses. SDS-PAGE analysis of the freeze-dried pellet and hydrolysates were carried out in 10-20% Tris-HCl ready gels as described in Section 3.2.3.2.

4.2.4. Particle size distribution of pellet hydrolysates

The volume weighted mean diameter (*d4,3*) of the fresh yolk, pellet and prepared fresh hydrolysates was measured without further centrifugation by laser light scattering particle size analyzer (Mastersizer 2000 S, Malvern). About 1 mL of sample was diluted in 10 mL MilliQ water and vortexed for about 20 s prior to analysis. The refractive index for water (dispersant) was 1.33 and for lipids chosen as 1.6 at 20 ^{o}C. Measurements were performed in duplicates from two independently hydrolysed pellet batches.

4.2.5. Cream separation and determination of total lipid and protein

The hydrolysates were divided into 50 mL tubes and centrifuged at 5 ^{o}C and 6,000×g for 30 min to accelerate the separation of cream and liquid phases. The subnatant phase was separated carefully by puncturing the bottom of the tube by a needle and collecting it in separate plastic tubes. All fractions were weighed and freeze-dried prior to further analysis. Determination of total lipid and protein and moisture contents was performed as described in Section 3.2.3.1.

4.2.6. Phospholipids analysis

4.2.6.1. Sample preparation

Total amount of lipid extract from freeze dried samples was dried under nitrogen and weighed. Then, the whole lipid extract was dissolved in 10 mL hexane in a volumetric flask. Sep-Pak Vac 6cc (1 g) silica cartridge column (Waters, Dublin, Ireland) was equilibrated with 10 mL chloroform and then 15 mL hexane: ethyl ether (1:1 v/v). Then, 0.5 mL of sample was placed in the cartridge to remove neutral lipids and collect the polar fraction. First, neutral lipids were removed using 25 mL hexane/ethyl ether (1:1, v/v). Then, polar fraction was eluted and collected using 15 mL methanol followed by 10 mL chloroform: methanol: water (3:5:2, v/v/v). Polar fractions containing PL were dried under a gentle stream of nitrogen. Then, samples were reconstituted in chloroform to a volume of 10 mL in a volumetric flask. About 25 µL of sample aliquots was diluted into 1.5 mL of acetonitrile: methanol (75:25, v:v) and then 5 µL was injected into the UPLC (Ultra Performance Liquid Chromatography). All solvents were HPLC grade (Fisher Scientific, Fair Lawn, NJ, USA).

4.2.6.2. Phospholipids quantification

For quantification, PL standards, purchased from Avanti Polar (Alabaster, AL, USA), were 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (16:0-18:1 PC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylethanolamine (16:0-18:1 PE) and 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphatidylcholine (16:0 LPC). PL in the samples were identified by comparison of their retention times to these commercial standards. PC, PE and LPC contents of samples were quantified using an ACQUITY^{TM} UPLC (Waters, Milford, MA, USA). For PL quantification, the Waters Acquity UPLC system equipped with a binary solvent manager, an Acquity column heater, a Waters Acquity UPLC BEH HILIC column (2.1 mm × 50 mm, 1.7 μm particle size) and an Evaporative Light Scattering Detector (ELSD) (ACQUITY UPLC ELS, Waters, Milford, MA, USA), operated with nitrogen as a nebulizing gas were used. A binary mobile gradient phase at a flow rate of 0.2 mL/min was applied. Eluent A was a mixture of acetonitrile and methanol (60:40 v/v), containing 5 mM ammonium acetate (99.1%, HPLC grade, Fisher Scientific). Eluent B was water plus 5 mM ammonium acetate. After injection, the sample was eluted with 100% solvent A for 3 min, increased to 5% eluent B for 3 min, followed by washing with 100% B for 5 min at a flow rate of 0.2 mL/min. The ELSD detector parameters were air pressure = 0.27 MPa, drift tube temperature = 70 ^{o}C, and gain = 500. A linear regression model was applied to obtain a linear equation for the plot of log peak area versus log quantity of each PL, which was then used for PL quantification. For validation of the PL quantification technique, the recovery of both PC and PE was determined by

spiking fresh yolk with PC (12.5-50 μg/mL) and PE (3.12-50 μg/mL) at a range of concentrations. Average recovery obtained was 90% (R-value=0.99) for PC and 45% (R-value=0.99) for PE. Coefficient of variation (CV) was 10% and 15% for PC and PE, respectively.

4.2.7. Emulsion preparation and characterisation

O/W emulsions were prepared and oil droplet size and coalescence index were measured as described in Section 3.2.4.1

4.2.8. Statistical analyses

Results of PL quantification and emulsion properties were subjected to general linear model, one-way analysis of variance (ANOVA) using GRAPHPAD PRISM (Graph pad software, San Diego, CA, USA) at a confidence interval of 95% ($p < 0.05$). Means were compared to determine significant differences at $p < 0.05$. Dunnet test was performed for comparison of PL recovery in creams against the pellet, while Tukey's HSD test was applied for the comparison of emulsion properties of the pellet and its hydrolysates.

4.3. Results and discussion

4.3.1. Hydrolysate properties

4.3.1.1. SDS-PAGE profile of leftover egg yolk pellet and its hydrolysates

Figure 4.2 shows high molecular weight SDS-PAGE profile of the pellet and its hydrolysates. The pellet had 22 protein bands, including some major bands as apoproteins at 223 kDa and 130-160 kDa. After enzymatic hydrolysis, these bands were hydrolysed into smaller fragments (Fig. 4.2). Hydrolysate of ProteaseP/LipaseAY30 (Lane c) showed less polydispersity and protein density

with a narrower molecular weight distribution compared to the other enzyme treatments.

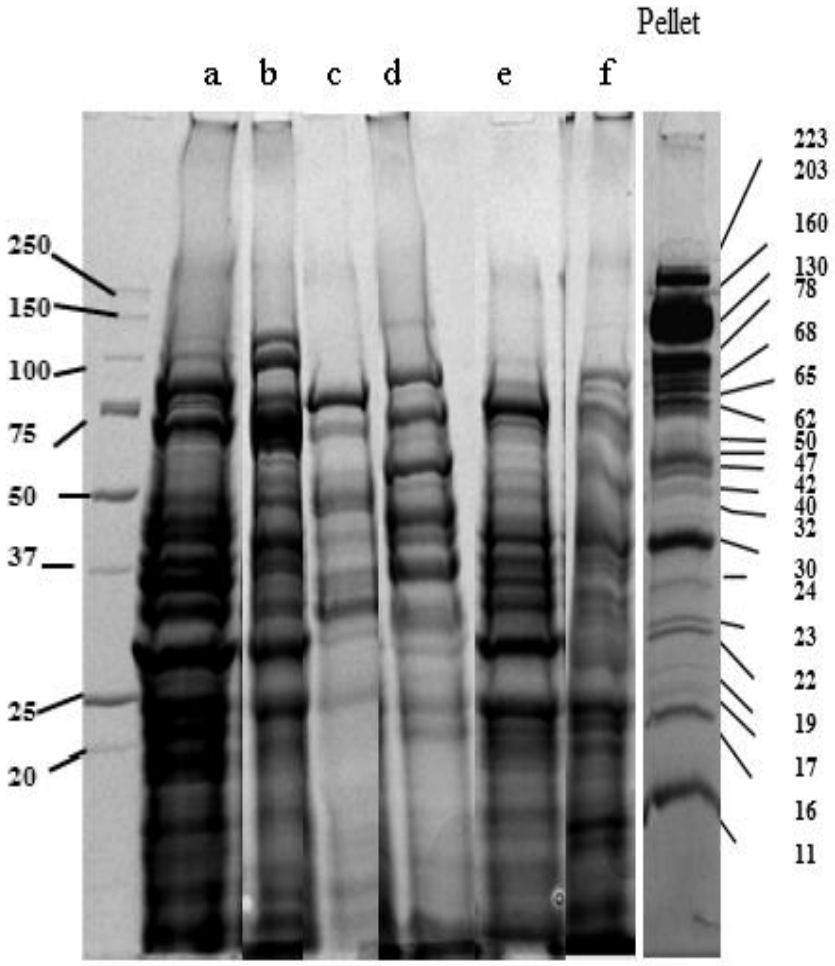

Figure 4.2. SDS-PAGE profile of the pellet and its hydrolysates in 10-20% Tris-HCl gel. (a) Protease II, (b) Protease M, (c) Protease P, (d) Protex 51FP, (e) Protease A, (f) Protex 7L and the pellet. All enzymatic treatments included Lipase AY30.

4.3.1.2. Particle size distribution of yolk, pellet and pellet hydrolysates

Egg yolk is an emulsion. Original egg yolk showed a monomodal particle size distribution with an average particle size (*d4,3*) of 2.2 μm (Fig. 4.3). The

average particle size of the leftover pellet increased to about 25.2 μm, along with a small peak a appearing between 100 - 1000 μm. The increase in the average particle size of the pellet could be due to the structural and rheological changes in the yolk after soluble proteins removal. After hydrolysis, the major peaks were shifted to the right and the average particle size increased for hydrolysed pellets, especially for the hydrolysates prepared with Protease P (121 μm) and Protease II (127 μm); however, the average particle size of Protex 51FP hydrolysate was decreased to 15 μm, with a smaller area for the peak between 100-1000 μm. Lipids of egg yolk are complexed to lipoproteins through non-covalent bonds. After hydrolysis of proteins by proteases as shown in Fig. 4.2, egg yolk still contained large molecular weight peptides, which may have interfacial activity.

However, depending on the enzyme type, the interfacial activity of proteins and other surface active compounds can be different. Partial hydrolysis of proteins may improve emulsifying properties through the exposure of functional groups (Guang and Wang, 2009). On the other hand, extensive hydrolysis of proteins results in small peptides may not form a strong viscoelastic film around oil droplets. Apoproteins from lipoproteins are large proteins with a flexible structure, which has been reported to be major surface active components from LDL and yolk (Anton et al., 2003).

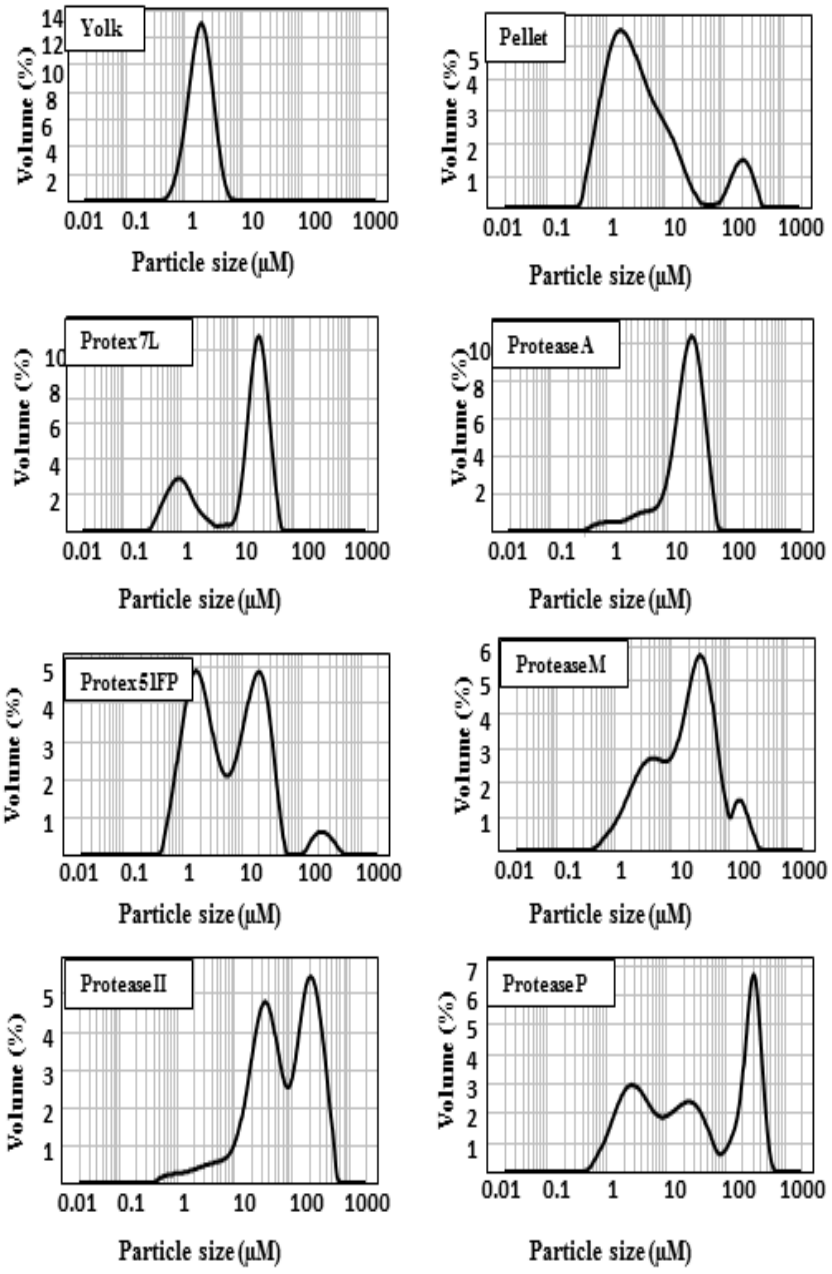

Figure 4.3. Particle size distribution profile of intact yolk, pellet and the hydrolysates of pellet

A complex between apoproteins and phospholipids on the surface holds triglycerides core and makes lipoprotein assemblies (Anton, 2007). Therefore, the degradation of apoproteins can have a remarkable impact on the instability of the pellet emulsion, which can be depicted as the increased average particle size of hydrolysates as shown in Fig. 4.3. The impact of combined proteolytic and lipolytic enzymes on emulsifying properties of egg yolk has been studied to a limited extent. For example, egg yolk proteins were solubilized and lipid from egg yolk was separated as a distinct oily phase from yolk after 6 h hydrolysis using a crude enzyme Newlase F (Amano), a mixture of lipase and proteolytic enzymes (Ohba et al., 1994). In this study, our trials with the currently available enzymes did not result in any considerable oil separation.

4.3.1.3. Cream composition

The outcome of the enzymatic hydrolysis of the pellet was a suspension with a tendency for phase separation, forming a cream phase on top and a liquid subnatant. About 20-35 g of cream was obtained from 50 g pellet, depending on the type of enzymatic treatment. The composition of the creams in terms of moisture, total lipid, protein and PL was shown in Table 4.1.

Fresh egg yolks used for the study contained about 16% protein and 32-34% lipid. Leftover pellet contained about 12% protein and 29% lipid. Mass balance calculation showed that 90% of total lipids from egg yolk were recovered in the pellet. Creams obtained after digestion were rich in lipid while the amount of proteins in their composition was reduced considerably. The creams obtained from Protease P treatment contained the least amount of protein and its subnatant

was a clear liquid, meaning Protease P had higher proteolytic activity and could solubilize more proteins. After centrifugation, a higher amount of dispersed particles were floating in the subnatant of Protex 51FP and Protex 7L and a precipitate was formed at the bottom of the tube for the subnatant of Protex 51FP. Therefore, some lipids remained in the subnatant of these treatments. The amount of lipid lost into the liquid phase after enzymatic digestion with Protease P, Protease M and Protease II was negligible. In fact, the subnatant of Protease P treatment was very clear and translucent.

Table 4.1. Protein, lipid and protein as well as PC, PE and LPC contents (%w/w) of the pellet, cream and subnatant from enzymatic hydrolyses of the pellet.*

	Pellet	Cream						subnatant					
		Protex 51FP	Protex 7L	Protease M	Protease P	Protease II	Protease A	Protex 51FP	Protex 7L	Protease M	Protease P	Protease II	Protease A
Moisture	57±0.3	69.2±2.2	71.7±1.9	65.6±6.4	47.7±6.2	58.8±8.7	58.6±4.9	96.6±0.6	97.6±0.5	97.6±1.6	98.2±0.2	98.8±0.5	98.2
Lipid	28.8±1.4	25.2±2.2	23.3±0.8	31±5.8	54.1±6.4	37±9.3	35.5±5.6	2±0.2	1.1±0.4	0	0	0	0
Protein	12.7±0.9	7.3±0.1	4.3±0.4	5.8±0.2	3.6±0.9	6.2±0.3	7±0.8	2.6±1.5	1±0.2	1.3±0.1	1.3±0.1	1.1±0.1	1.4
PC	7.1±1.2	1.6±0.3	2±0.3	2.2±1	10.3±3.1	8.4±3.6	7±1	0.8±0.2	0.7±0.1	0	0	0	0
PE	2.5±0.8	0.4±0.1	0.4	0.6±0.2	3.2±0.07	1.8±1	1.2±0.6	0.3±0.1	0.4±.2	0	0	0	0
LPC	N.D.	N.D.	N.D.	2.8±0.2	0.7±1	N.D.	0.9	N.D.	N.D.	0	0	0	0

*Mean± standard deviation of two independently hydrolysed pellet batches (n=2). Final composition of samples was reported on wet basis (N.D. not detected).

Enzymatic treatment, depending on the type of protease applied, seemed to have prominent effect on the PL composition of hydrolysates (Table. 4.1 and Fig. 4.4). Egg yolk contains about 5% lyso-ethanolamine (LPE) and LPC in its PL composition (Kovacs-Nolan et al., 2005). Despite the fact that LPC was not detected in the original yolk or the pellet due to the detection limit of the analytical system, after digestion by Protease M, Protease P and Protease A, which also included Lipase AY30 as part of the treatments, the amount of LPC in the creams obtained was high enough to be detected by the system. The highest conversion of PC to LPC was for the treatments of Protease M (Table 4.1 and Fig. 4.4). Pellet hydrolysis with Protex 7L and Protex 51FP along with Lipase AY30 considerably decreased the PC and PE content in both cream and subnatant fractions. No LPC was detected after these treatments, meaning the pellet treatment with Protex 7L or Protex 51FP degraded considerable amounts of PC and PE. A shift in the retention time of LPC peak in the egg yolk and creams was probably due to the difference in the fatty acid compositions of egg yolk sample, creams (obtained after lipase treatment), and the synthetic one used as analytical standard.

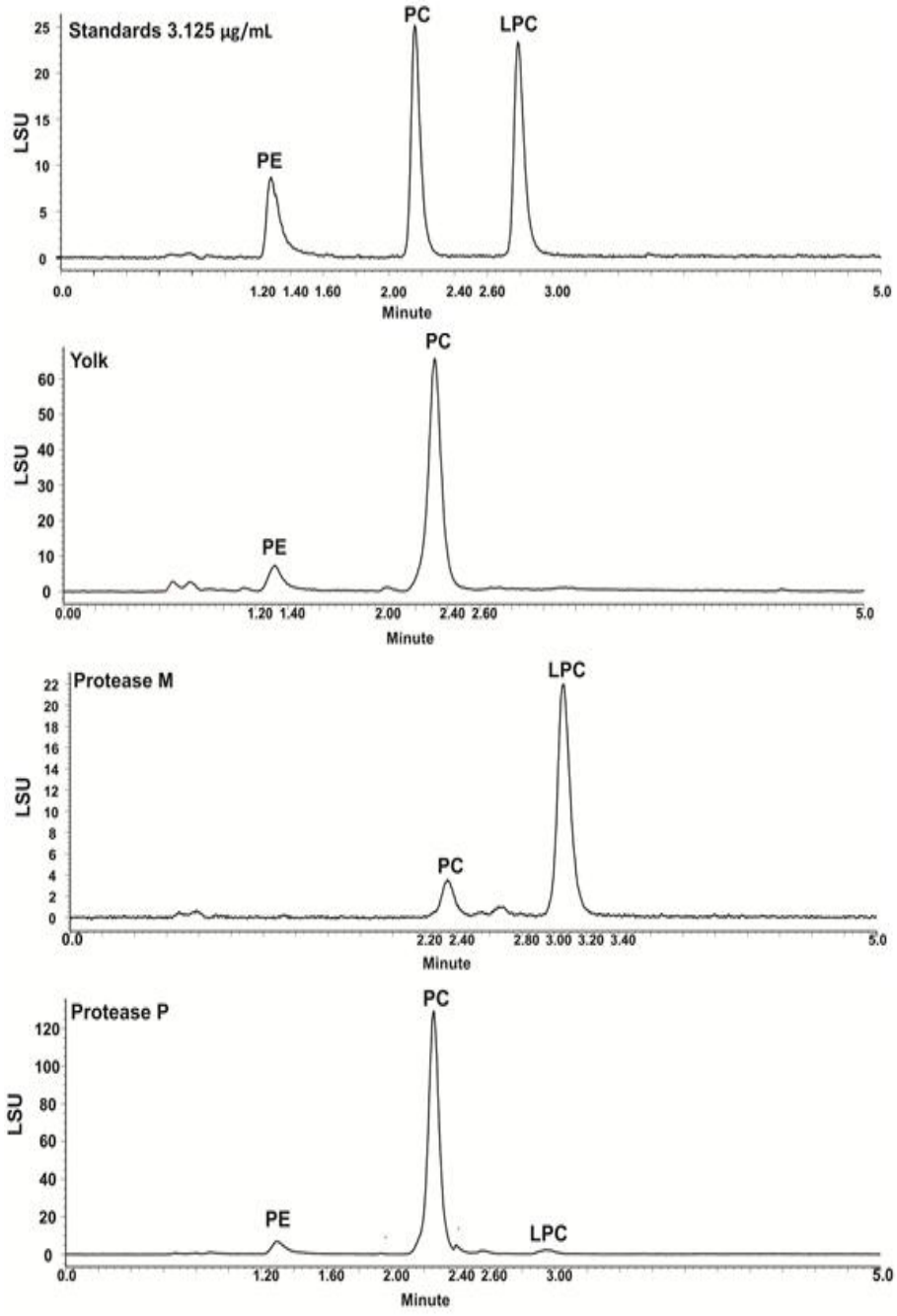

Figure 4.4. Phospholipid chromatograms (PC: Phosphatidylcholine, PE: Phosphatidylethanolamine and LPC: lysophosphatidylcholine) of yolk and creams obtained after digestion with Protease P, Protease M. All enzymatic treatments included Lipase AY30.

Figure 4.5 shows PL recovery in the pellet and creams based on the original amounts of yolk used for the process. Over 90% of PC and PE from egg yolk were recovered in the pellet. After enzymatic hydrolyses and centrifugation, PC and PE recovery in the creams from Protex 7L, Protex 51FP, Protease M and Protease A significantly decreased and was the lowest for creams of Protex 51FP, Protex 7L and Protease M. The highest recovery of PC was for Protease P followed by Protease II, where 81% and 77% of PC from the original yolk was recovered in the creams, respectively. The recovery of PE in cream was lower than that of PC for all enzymatic treatments. The drastic decrease of PL recovery in the creams for Protex 7L and Protex 51FP was partially due to some lipids lost in the subnatant but mostly due to the extensive PL conversion after enzymatic treatment with these enzymes as shown in Table 4.1. Extensive PC to LPC conversion occurred for Protease M and to a lesser extent for Protease A and Protease P treatments, all including Lipase AY30 (Table 4.1 and Fig. 4.4).

Although lipases such as Lipase AY30 target triglycerides as their substrates, they may also hydrolyse PL. However, their activity ratio towards triglycerides was reported to be hundred times higher than their specificity on PL (Haas et al., 1995). It was interesting that the PC conversion to LPC was not at the same rate for all enzymatic treatments. A peak related to LPC was observed for Protease II and Protease A, and it reached the highest level for the cream of Protease M. However, no LPC was detected for Protex 51FP or Protex 7L treatments. Whether the cleavage of apoproteins with some specific proteases

could favour the exposure of PC and generally PL to enzymes and make it an accessible substrate for the lipase needs further confirmation.

A high error bar observed for PE and total PL recovery from cream of Protease P + Lipase AY30 could be due to a higher rate of PE degradation after enzymatic digestion or through sample preparation during analyses.

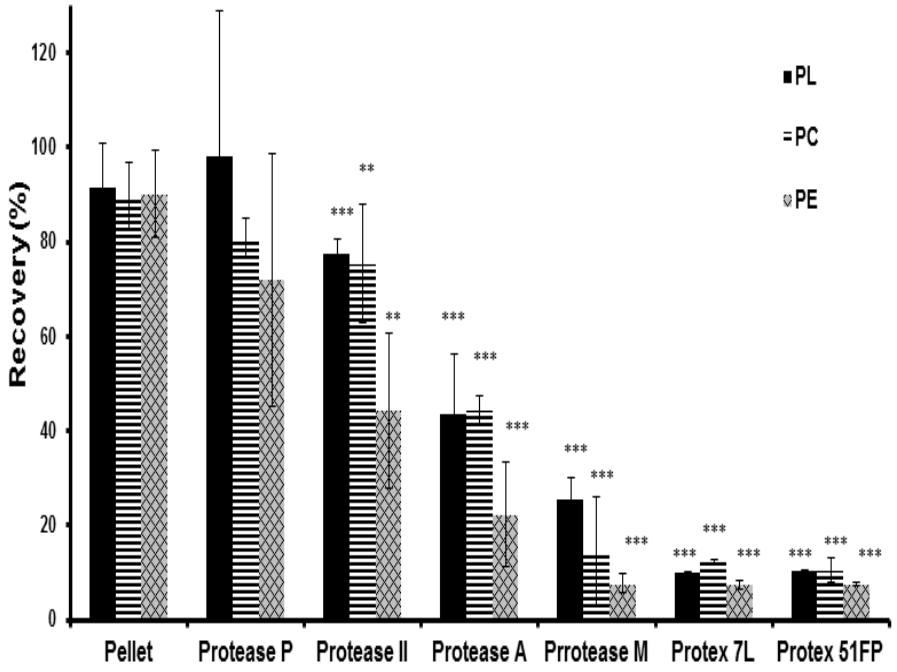

Figure 4.5. Recovery of two major PL classes (PC and PE) and total (PC+PE) or (PC+PE+LPC) in the pellet and its creams obtained after enzymatic hydrolysis (n=2) based on the amounts of PL in the original yolk. All enzymatic treatments included Lipase AY30. Bars marked with (*, **, ***) are significantly different from those of the pellet at $p < 0.05$, 0.01, 0.001, respectively.

4.3.2. Emulsion properties of hydrolysates

This study demonstrated that after enzymatic treatments, the average particle size and distribution of hydrolysates increased compared to those of the intact yolk and the pellet. In this part, emulsion properties of hydrolysates were investigated in terms of oil droplet size and tendency of the emulsion to coalescence instability. Figure 4.6 shows the average oil droplet size of the emulsions prepared with the pellet and its hydrolysates.

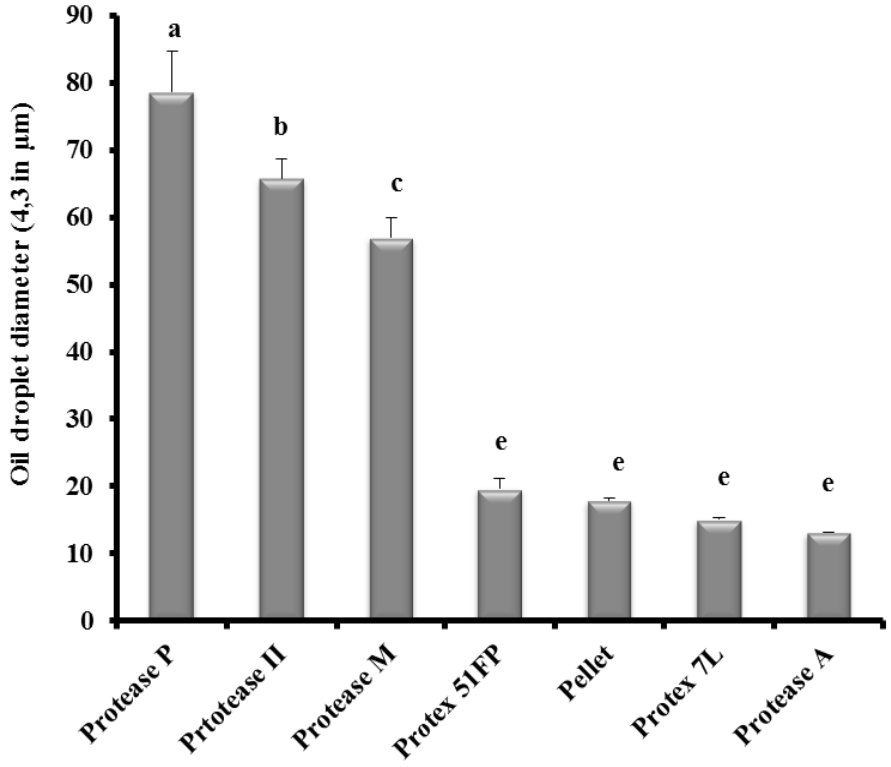

Figure 4.6. Mean volume weighted diameter of oil droplets of emulsions prepared from the hydrolysates of six enzymatic treatments (n=3). All enzymatic treatments included Lipase AY30. Bars with different letters are significantly different at $p < 0.05$.

Emulsions prepared with the leftover egg yolk pellet had an average oil droplet size of 17 μm. The average oil droplet size of the emulsions prepared with the hydrolysates of Protease P, Protease II or Protease M were significantly ($p < 0.05$) higher (78, 65, 56 μm, respectively), while those of Protex 51FP, Protex 7L and Protease A were close to that of the pellet ($P > 0.05$). Emulsions with larger oil droplets are generally prone to coalescence instability. A similar trend of increase in the average size of oil droplet was also observed for coalescence indices (Fig. 4.7) with the exception of Protex 51 FP where its coalescence index was significantly higher ($p < 0.05$) than that of the pellet.

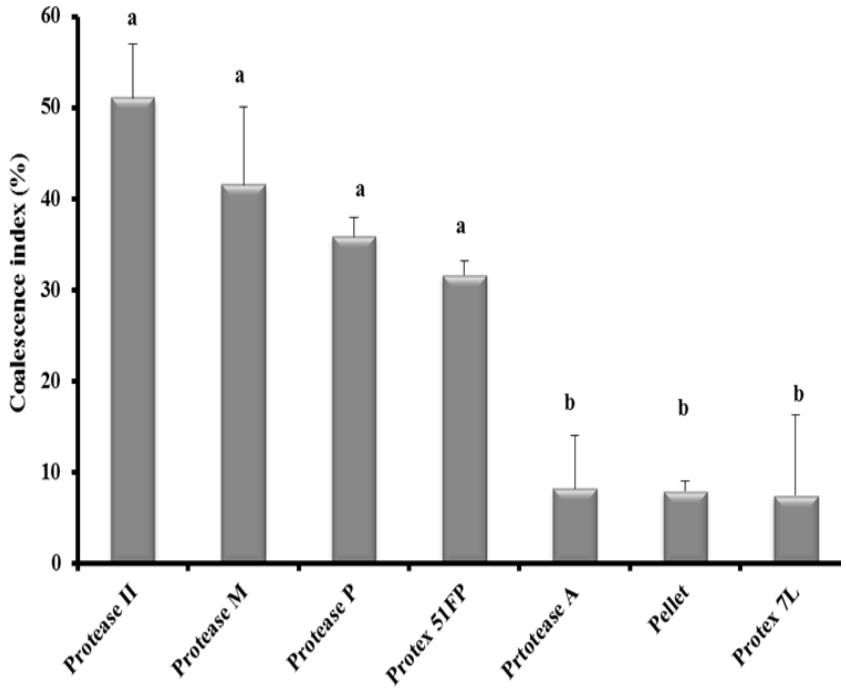

Figure 4.7. Coalescence index of emulsions prepared from the hydrolysates of six of six enzymatic treatments (n=3). All enzymatic treatments included Lipase AY30. Bars with different letters are significantly different at $p < 0.05$.

The largest oil droplet size was for the emulsions prepared with Protease P hydrolysate. Protease P is specified as a highly active proteolytic and peptidase enzyme while Protex 7L was a peptidase with a lower enzyme activity. As shown in Fig. 4.2, Protease P hydrolysate showed less peptide bands and its cream also contained lower protein content compared to those of other enzymes, indicating Protease P hydrolysate is composed of mainly small peptides that could not be detected in SDS-PAGE. Proteases can change the size of proteins, modify their hydrophobic and charge properties; thus their functional properties as well (van der Ven et al., 2001; Guang and Wang, 2009). The emulsion property of a food protein is determined by its size, charge, the balance of hydrophobic and hydrophilic side chains, rigidity and flexibility (McClements, 2005).

Despite the fact that PL were greatly degraded after the pellet treatments with Protex 7L, Protex 51FP or Protease A, these hydrolysates showed good emulsifying properties since the average oil droplet size of emulsion prepared from these treatments was as small as that of the pellet. This may to some extent indicate that the proteins may have a higher impact than PL on the emulsifying properties of the pellet and its hydrolysates. PL are smaller molecules compared to proteins and probably proteins and large peptides still played a greater role in emulsion stability. Other researchers claimed that proteins especially apoproteins from LDL play a significant role in egg yolk emulsion properties and its stability (Mizutani and Nakamura, 1987; Anton and Gandemer, 1997). Bringe et al. (1996) showed that apoproteins, compared to PL, are larger molecules with a flexible structure. Flexible proteins can easily unfold and cover oil droplet surface and

their steric hindrance prevents aggregation between droplets (Dickinson, 1994). Due to their high molecular weight, proteins form a stronger viscoelastic interfacial film around the oil droplets and stabilise them against coalescence (Chen and Dickinson, 1998). The change in triglyceride composition and the amount of lipid hydrolysis products, including glycerol, free fatty acids, mono- and diglycerides, formed after lipase treatment were not analysed in this study. Therefore, it is not possible to assess the effect of triglyceride hydrolysis products on the emulsifying properties of final hydrolysates. Better characterisation of this aspect requires further research.

Therefore, the results of the current study may suggest that the hydrolysis of proteins to smaller molecules affects the emulsion stability more than lipids. From a technological point of view, creams obtained from Protease P and Protease II in combination with Lipase AY30 could be the best candidates as the starting material for PL extraction using SC-CO_2 technology, based on two considerations: a) the highest recovery of PC in the cream fractions obtained from these treatments, b) the lowest emulsifying property of these treatments, meaning the bonding forces between lipids and proteins are weaker.

4.4. Conclusions

The findings of this study demonstrated that enzymatic treatments (proteases + Lipase AY30) could change the physicochemical properties of the leftover pellet. The average oil droplet size was higher for emulsions prepared from Protease P hydrolysates. Emulsions prepared from Protease II, Protease P and Protease M hydrolysates were prone to coalescence instability compared to

the emulsions prepared from the pellet or hydrolysates of Protex 7L and Protease A. The particle size distribution of hydrolysates also changed depending on the applied enzyme, where the average particle size was highest for Protease II and Protease P hydrolysates.

Over 80% of PL from the yolk was recovered in the cream from Protease P. From the technological point of view, creams with the least emulsion stability and highest phospholipid content would be suitable for further de-emulsification and extraction studies. Therefore, cream fraction from Protease P or Protease II hydrolysates may be used to develop "green" technologies such as SC-CO_2 extraction of PL.

4.5. References

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5. Effect of enzymatic hydrolysis on the extractability of phospholipids from leftover egg yolk using supercritical CO_2[3]

5.1. Introduction

As already discussed in previous chapters egg yolk has great amounts of high-value proteins and phospholipids (PL). About one-third of egg yolk lipid composition is PL with a high content of about 70% phosphatidylcholine (PC) (Nielsen and Shukla 2004; Kovacs-Nolan et al. 2005), which has broad applications in pharmaceutical and nutraceutical products and are routinely added into infant formula (Zeisel 1992; Rader et al. 2004). After IgY isolation through 10-fold dilution, average 90% of PL remains in the pellet, which can be used for high-value PL extraction.

For large scale production of PL, egg yolk lipids are usually extracted and fractionated using organic solvents such as acetone, dimethyl ether, hexane and ethanol (Schneider 1989; Nielsen and Shukla 2004; Palacios and Wang 2005a,b; Gadkowski et al. 2012). In particular, the deoiling process (fractionation of non-polar and polar lipids) involves the use of a large amount of harmful hexane.

Supercritical carbon dioxide (SC-CO_2) technology has been introduced to food industry over the past three decades with achievements at industrial scale as a "green" technology for the extraction of bioactive components and lipids from different natural sources (Temelli 2009). Due to being inflammable, abundant,

[3] A version of this chapter has been submitted to Journal of Food Engineering for publication.

non-toxic and providing an oxygen-free environment during extraction and no solvent residue left in the product, SC-CO_2 has been an attractive solvent for the extraction of lipids and other bioactive compounds from various natural matrices (Bulley et al. 1992; Temelli 1992; Dunford and Temelli 1995; Montanari et al. 1996; Herrero et al. 2006; Sahena et al. 2009; Temelli 2009). However, due to the polar nature of the PL, the extraction of PL using SC-CO_2 requires a polar co-solvent to be added into the CO_2 stream. Considering thermodynamic and food safety aspects, ethanol as a GRAS (generally regarded as safe) solvent is commonly used as a co-solvent for PL extraction (Rossi et al. 1990; Dunford and Temelli 1995; Catchpole et al. 2009).

Scarce-solubility of PL in the neat CO_2 has been applied as an advantage for deoiling of PL. Dunford and Temelli (1995) and Montanari et al. (1996) used a two-step extraction process for PL recovery from soybeans. In the first step, neutral lipids were removed from feed using neat CO_2, and then concentrated PL was extracted by injecting ethanol into the CO_2 stream in the second step. Several studies have reported deoiling or PL extraction from dried egg yolk using SC-CO_2. Froning et al. (1990) removed the majority of cholesterol and neutral lipids from egg yolk using neat CO_2. Bulley et al. (1992) investigated the removal of cholesterol and triglycerides from freeze-dried egg yolk at pressures ranging from 15 to 36 MPa and temperatures of 40 to 75 ^{o}C. PL could be extracted along with the neutral lipids after 3-5% methanol or ethanol addition to SC-CO_2. Wu and Hu (2000) developed a mathematical model for oil extraction from egg yolk using neat CO_2. Boselli and Caboni (2000) reported extracting of PL from dry egg yolk

at micro-scale using neat CO_2 at 51.7 MPa and a density of 1.06 g/mL of CO_2 at 40 ^oC temperature. Extraction was performed in four cycles, each being 30 min. Total PL extraction yield was reported to be 17.7 mg/100 mg of dry yolk, which was comparable to that obtained by solvent extraction according to such as Bligh and Dyer (1978). Boselli and Caboni (2000) suggested two possible reasons for the extraction of PL using neat CO_2. First reason could be due to the higher density of CO_2 at the condition employed. Secondly, they believed that freezing-thawing process carried out during each cycle of depressurization of extraction might have caused destruction of lipid-protein aggregates, thus making polar lipids more extractable. Later, Elts et al. (2003) tried to extract PL using neat SC-CO_2 from dry yolk in 4 cycles at pressures of 25 MPa and 51 MPa at 40 ^oC. However, no considerable amount of PL (less than of 1% of PL content of egg yolk) could be extracted using neat CO_2. In the next part of the same study, PL from dry egg yolk was extracted using ethanol. After drying the extracted PL were mixed with the egg yolk protein residue to investigate whether bonding between PL and proteins was a barrier for PL extraction using neat SC-CO_2. The mixture was subjected to neat CO_2 extraction and 51 MPa at 40 ^oC using 200 g of CO_2. The amount of extracted PL was negligible, showing that polar PL cannot be extracted in without a polar co-solvent. PL could be extracted only after the addition of at least 5% ethanol. It was reported by Elst et al. (2003) that the majority of PL from dry yolk could be extracted using CO_2 at 51 MPa and the presence of 15% ethanol and 40 ^oC.

Shah et al. (2004) extracted 49 g PC/kg of dried yolk using the two step extraction technique using SC-CO_2+ethanol. The second step of extraction was performed for 1 h at 41.1 MPa, 60 ^oC and flow rate of 5 L/min (reported at 0.1 MPa and 25 ^oC) and using 50% ethanol. However, the purity of PC in the extracts obtained was not reported. More recently, Aro et al. (2009) studied PL extraction from dried egg yolk using supercritical anti-solvent (SAS) property of CO_2. In the first part of the study, dried egg yolk was dissolved in ethanol and stored at 4^oC for 24 h. Then, the ethanolic solution containing PL was concentrated using a rotary evaporator and then pumped into the high pressure CO_2 chamber. The overall recovery of PC+PE with this method was about 40-50% with a high purity of PL ranging from 72-99%. In the second part of their study, Aro et al. (2009) extracted PL using the two-step extraction process, first by deoiling dry egg yolk with neat CO_2, then extracting PL using SC-CO_2+ethanol. The extract from the second step was then injected into the SAS system. Due to the low recovery of PL during the two step extraction (12-18%), extracts from two separate batches were combined for the SAS process after which a very high purity PL (99%) was obtained.

Despite all the achievements summarized above, PL extraction from dry egg yolk had low recovery, even in the presence of polar solvents such as ethanol due to the specific structure and position of PL in egg yolk, where PL are attached to apoproteins through non-covalent bonds (Burely and Vadehra 1989). Pretreatment of egg yolk using enzymatic hydrolysis may be a viable approach to improve the recovery of PL during SC-CO_2 extraction. The proteolytic enzymes

hydrolyse egg yolk proteins including apoproteins into smaller peptides, while lipases could hydrolyse the triglyceride core of lipoproteins, thus facilitate destruction of the lipoproteins assembly and release the lipids. This may weaken the overall structure of the yolk and facilitate diffusion of SC-CO_2 into the hydrolysed matrix and increase the overall PL extractability. To the best of our knowledge, there have been no studies investigating the effect of enzymatic hydrolysis and structural changes of the egg yolk on the extractability and recovery of PL.

Therefore, the objective of this study was to investigate the effect of enzymatic treatments on the overall extractability and purity of PL, especially PC as the high-value compound, from egg yolk and the pellet using the two-step SC-CO_2 extraction method. In the first step, neutral lipids were extracted from the samples using neat CO_2. In the second step, ethanol was injected into the SC-CO_2 to extract PL. Recovery and purity of extracts obtained from the hydrolysed pellet were compared to those from dry yolk and pellet.

5.2. Materials and Methods

5.2.1. Egg yolk pellet preparation

Egg yolk fractionation and pellet preparation was performed as described in Section 3.2.1.

5.2.2. Enzymatic treatment and cream preparation

For enzymatic treatments, the fresh pellet was diluted 6-fold with distilled water and mixed for 1 h using a magnetic stirrer to obtain a homogenous slurry, and then hydrolysed by a combination of Lipase AY 30 and one of the two proteases: Acid Protease II and Protease P. Enzymatic hydrolysis conditions and cream preparation were performed as described in Section 4.2.2 and 4.2.5. Creams were freeze dried and stored at -20 ^{o}C until for SC-CO_2 extraction.

5.2.3. Proximate analyses

Lipid and protein contents of freeze-dried samples were determined according to the protocols described in Section 3.2.3.1. Moisture content was determined gravimetrically at 105 ^{o}C with measurements taken over 16 h, until constant weight was reached.

5.2.4. SC-CO_2 extraction process

The laboratory scale supercritical fluid extraction system described previously by Dunford and Temelli (1995) was used for the extraction process. The flow diagram of the unit is shown in Figure 5.1. Treatments for extraction were freeze-dried yolk, pellet and creams of the two enzymatic treatments as described above. About 2-3.5 g of dried sample was mixed with 150 g of glass beads (3 mm, Fisher scientific, Pittsburgh, PA, USA) and filled into a stainless steel basket to be placed into the extraction vessel. Dry yolk and pellet were crushed into the powder form using mortar and pestle. Glass wool was placed at both ends of the basket before closing the caps of the basket. Lipids from samples were extracted in two steps at 48.3 MPa (the highest possible pressure that can be

reached in this extractor), 70 ^{o}C temperature and CO_2 (99.9 % (wt%) pure, bone dry, Praxair Inc., Edmonton, AB, Canada) at a flow rate of 1 L/min (measured at ambient conditions). These conditions were chosen to maximize the solubility of lipids in SC-CO_2 within the system. In the first step, neutral lipids were extracted using neat CO_2 until no more extract was collected.

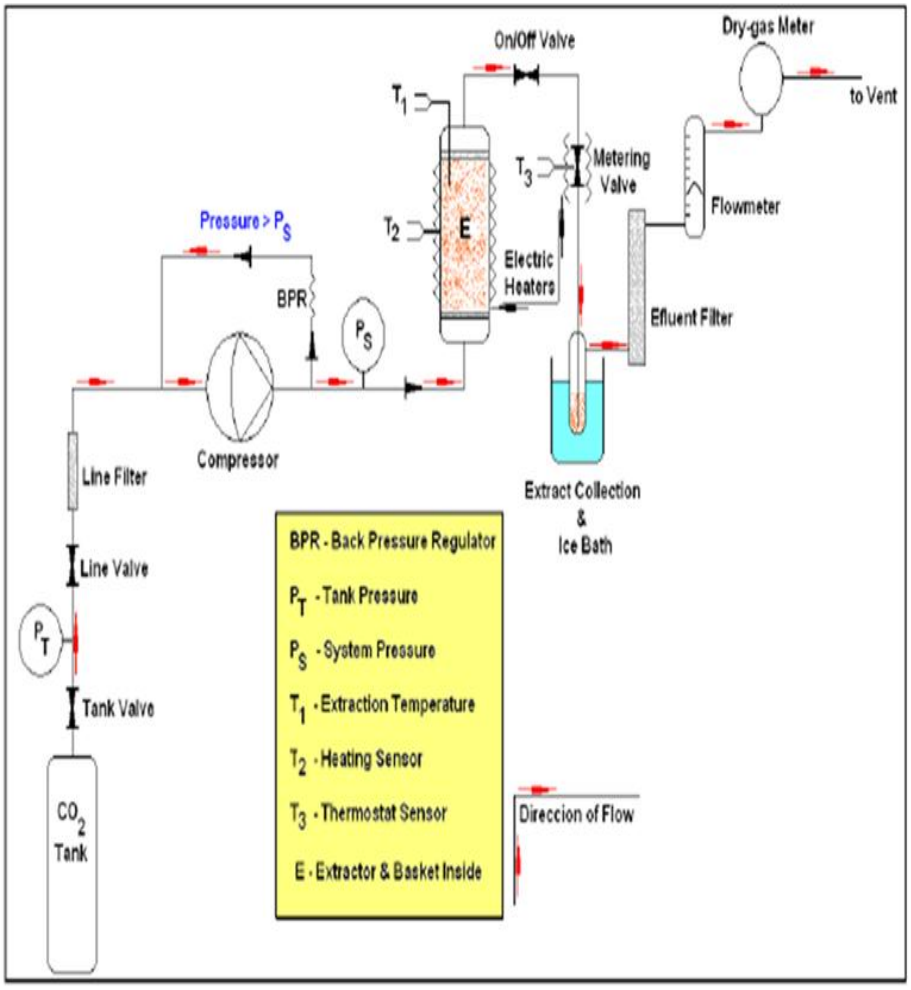

Figure 5.1. Flow diagram of the supercritical fluid extraction unit.

Then, in the second step of extraction, anhydrous ethanol was injected into the CO_2 line prior to entry into the extraction vessel using a high pressure piston pump (Model 305, Gilson Inc., Middleton, WI, USA) at a flow rate of 0.2 mL/min in order to get a molar fraction of 8% ethanol in CO_2. Polar lipids were extracted at this step for about 6 h. In the first part of the extraction using only neat CO_2, extract fractions were collected at each 20 L of CO_2 consumption, except for the pellet where the extract collection was performed at every 40 L of CO_2. When the extraction curve reached a plateau, ethanol injection was initiated. For the second part of extraction with ethanol injection, extracts were collected every 20 L consumption of CO_2 for the first six fractions. After that extracts were collected every 40-60 L of CO_2 consumption. Extracts from CO_2+ethanol steps were dried under a gentle stream of nitrogen at 50 ^{o}C until they were completely dry and reached constant weight. Schematic presentation of the whole process for different treatments and SC-CO_2 extraction has been demonstrated in Figure 5.2. Extracts were collected in glass tubes attached to the depressurization valve and placed inside an ice bath. After each extract collection, tubing attached to the depressurization valve was washed with chloroform. The washings collected in a test tube was then dried at 50 ^{o}C under a gentle nitrogen stream until its weight reached a constant level and recorded for plotting the extraction curve together with the extract fractions.

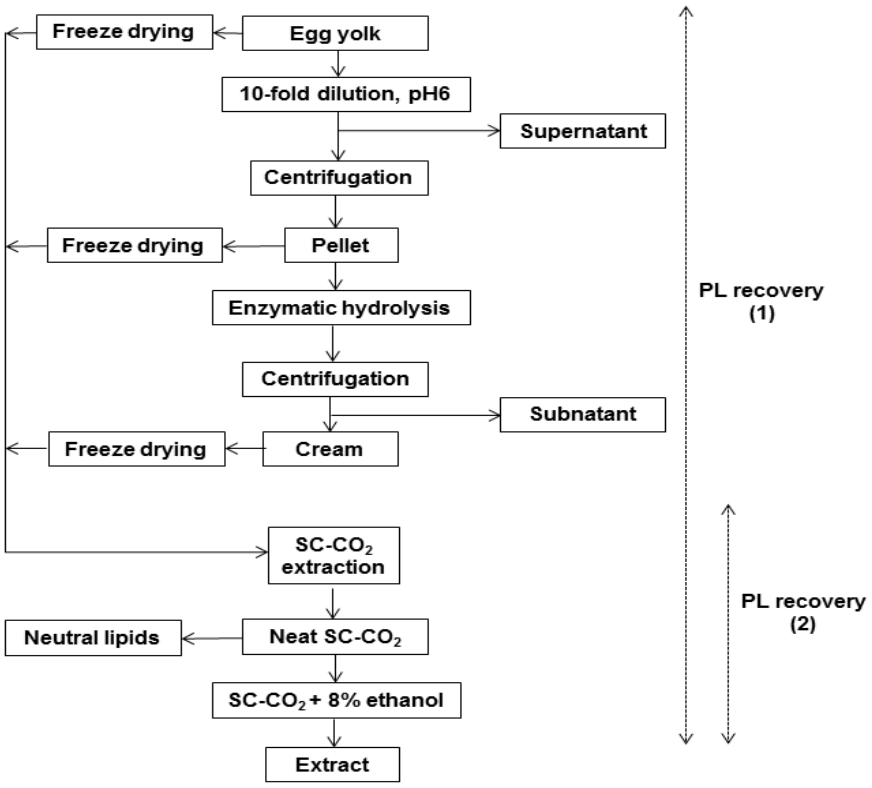

Figure 5.2. Schematic presentation of the whole process for different treatments and SC-CO_2 extraction. PL recovery (1) is based on yolk and PL recovery (2) is based on the feed material used for SC-CO_2 extraction.

5.2.5. Phospholipids analysis

5.2.5.1. Sample preparation

Lipid samples for PL analyses using organic solvents were prepared from freeze-dried yolk, pellet and yolk samples as described already in Section 3.2.3.1. Lipids were dried under nitrogen and weighed. Extract fractions obtained from each neat CO_2 or CO_2+ethanol steps collected over time were combined and then used for PL analyses. Then, the total lipid extracts were fractionated and prepared for injection as described in Chapter 4.2.6.1.

5.2.5.2. Phospholipids quantification

PL were quantified according to the procedure described in Section 4.2.6.2. The purity (%) of the extracts was estimated by measuring the amount of PC or PL in the extract obtained from SC-CO_2+ethanol step divided by the total mass of the extract obtained by SC-CO_2+ethanol as follows:

PL (or PC) purity (%) = [PL (or PC) amount in extract (g) / total amount of extract (g)]×100

The recovery of PC and total PL in the SC-CO_2 extracts was calculated by comparing the amounts of PC and total PL extracts to those amount that could be extracted using organic solvents from original egg yolk or the feed material used for SC-CO_2 extraction. In the first recovery calculation (yolk based, PL recovery (1) in Fig. 5.2), the amount of the initial egg yolk required for the whole process and its PL content was considered. Therefore, the recovery was the amount of PL in the SC-CO_2 + ethanol extract divided by the PL amount in the initial yolk used for the process.

PL(or PC) recovery (yolk-based) = [PL (or PC) amount in extract (g) per gram of cream / PL amount (g) in yolk needed to produce 1 g of cream)]×100

In the second approach (PL recovery (2) in Fig. 5.2.), to understand the structural impact after pellet hydrolysis on the extractability of PL and the efficiency of SC-CO_2 + ethanol extraction, the recovery was calculated based on the amount of PL present in the cream used as the feed material for supercritical extraction.

PL (or PC) recovery (based on SC-CO_2 extraction feed) = [PL or (PC) amount in extract (g) per gram of cream/PL amount (g) in 1 g of cream]×100

5.2.6. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The SDS-PAGE analysis of the freeze-dried creams was carried out in 10-20% Tris-HCl ready gels as described at 3.2.3.2.

5.2.7. Statistical analyses

All SC-CO_2 extractions were performed in triplicates, except for the dry pellet which was in duplicates. Treatments for statistical analyses were yolk, pellet, and creams from Protease P and Protease II (both enzymatic treatments included Lipase AY30), all freeze dried. Results of PL recovery and purity were subjected to general linear model, one-way analysis of variance (ANOVA) using GRAPHPAD PRISM (Graph pad software, San Diego, CA, USA) at a confidence interval of 95% ($p < 0.05$). Means were compared against yolk using Dunnet test to determine significant differences.

5.3. Results and discussion

5.3.1. Composition of dry yolk, pellet and creams

Total lipid and PL composition are presented in Table 5.1. After fresh pellet hydrolysis by enzymes, a biphasic system with a cream phase rich in lipid on the top and a clear subnatant liquid was formed for the Protease P treatments, while the subnatant phase from Protease II was not as translucent as that from Protease P treatment. After enzymatic digestion, some proteins were hydrolysed into smaller peptides and solubilized into the subnatant liquid phase, while the lipids remained in the cream. The dry yolk and pellet contained about 31% and 28% protein, while dry creams were lower in protein (12% and 17% for Protease P and Protease II, respectively) and rich in lipid (Table 5.1).

Table 5.1. Total lipid and major PL composition (g/100 g dry matter) of feed materials used for SC-CO_2 extraction.

Treatment	Lipid	PC	PE	LPC
Yolk	65±2	14±1	5±2	N.D.
Pellet	70	16±2	6±1	N.D.
Cream-Protease P	86±3	16±2	5±0.9	2±2
Cream-Protease II	82±0.5	19±0.5	2±0.5	2.1

N.D. not detected

According to Kwan et al. (1991) and the results from this thesis research (Chapter 3), after 10-fold dilution of yolk and separation of the supernatant, 90% of lipids from egg yolk remained in the pellet after soluble proteins removal. After hydrolysis, the lipid concentration in dry matter increased while protein content was lower for creams. PC and PE were the major PL in all treatments. While no

LPC was detected in yolk and pellet, about 2.5% and 2.1% of creams composition obtained with Protease P and Protease II were LPC, respectively. LPC makes up about 3-6% of PL composition in egg yolk (Rossi, 2007). In this study, however, no LPC could be detected in the yolk due to the detection limit of the analytical system. After enzymatic treatments, it seemed that Lipase AY30 could convert a small portion of PC to LPC, which increased the total LPC concentration to the detectable level. PE concentration decreased considerably in the cream obtained with Protease II. It is possible that some PE was converted to LPE due to the Lipase AY30 activity; however, since PC was the major target of this study, only LPC content was quantified.

5.3.2. SDS-PAGE profile

High molecular weight protein profile of the yolk and the cream fractions are shown in Figure 5.3. The largest peptide fraction for cream of Protease P was about 75 kDa, while for SDS-PAGE analyses of cream fractions and their comparison to the yolk showed that the major proteins from the yolk with molecular weights of 203, 160, 130, 90 kDa were hydrolysed into smaller fractions (Fig. 5.3). The largest protein fraction of hydrolysate from Protease II was around 110 kDa. Our results in the previous chapters and here demonstrate that Protease P had higher proteolytic activity than Protease II, since it could hydrolyse the pellet proteins to smaller peptides. The higher hydrolysis degree can facilitate separation of lipids attached through non-covalent bands to proteins. Also, it may improve the solvent diffusion and therefore increase lipid solubility in SC-CO_2+ethanol.

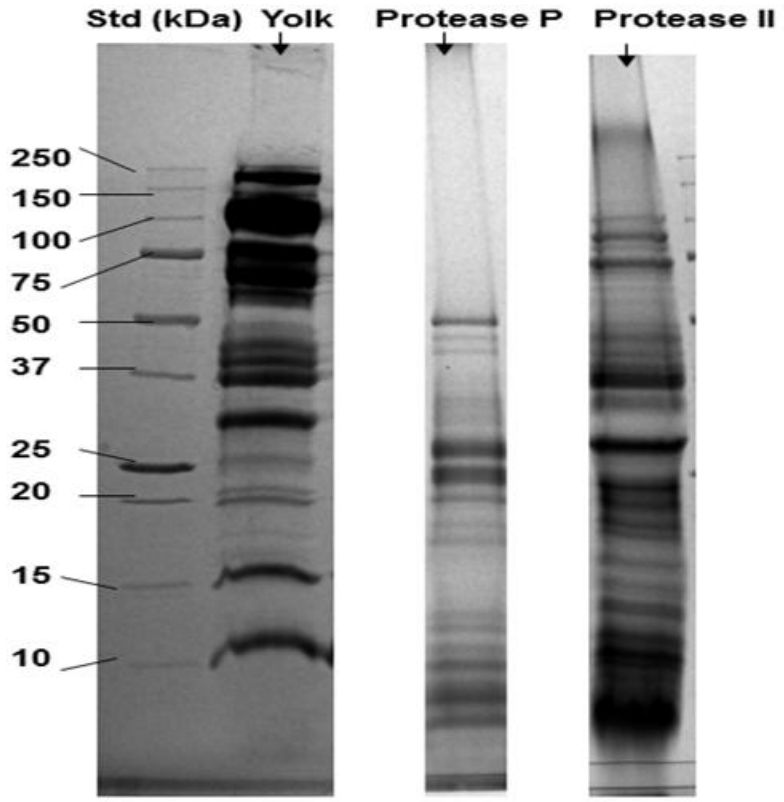

Figure 5.3. SDS-PAGE profile of yolk and its cream after hydrolyses with Protease P and Protease II; both enzymes treatments included Lipase AY30.

5.3.3. SC-CO_2 extraction curves

The extraction curves for yolk, pellet and creams were obtained and plotted over two steps of extraction involving neat CO_2 followed by CO_2+ethanol (Fig. 5.4).

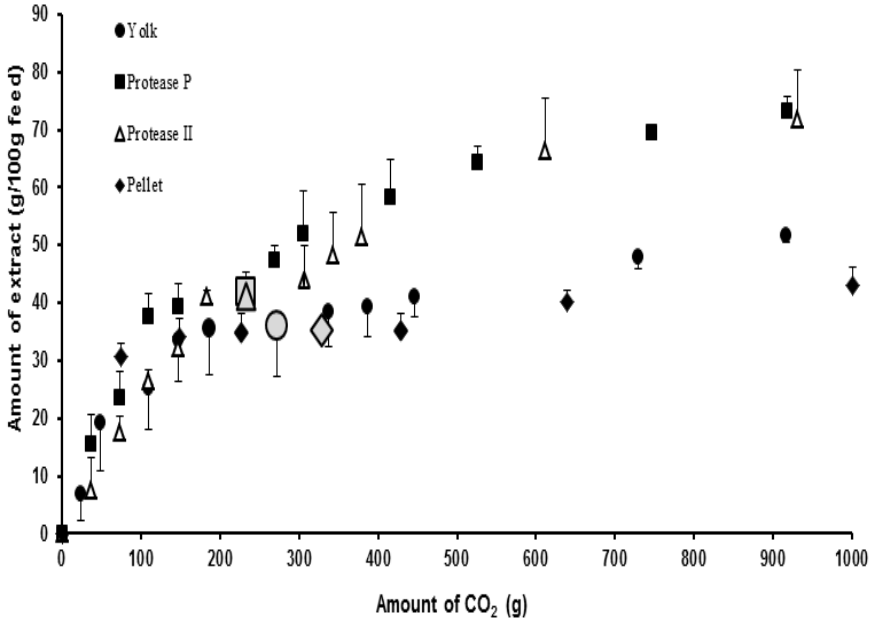

Figure 5.4. Total extract yield trend of different treatments using the two-step SC-CO_2 extraction process (n=3, for the pellet n=2). Larger gray symbols show the start of ethanol injection.

For all extractions, as expected there was a gradual increase in the mass of extract collected, establishing the typical initial linear region with easily accessible lipids being solubilized in the neat CO_2. After a certain period of time, no more material could be extracted using neat CO_2 and the extraction curve reached a plateau, where extraction was limited by diffusion. Then, ethanol addition into SC-CO_2 as co-solvent was initiated as indicated with the larger grey symbols in Figure 5.4.

After ethanol injection into the SC-CO_2 stream, another increase in extraction yield was observed, corresponding to solubilisation of PL and probably other ethanol-soluble compounds such as peptides. Total extract yield for Protease

P (73.2%) and Protease II (71.8%), which contained a higher amount of lipids, was higher than that for dry yolk (52%) and pellet (43%).

CO_2 loading (g extract/g CO_2) or slope of the initial linear portion of the extraction curves obtained during the first step of extraction with neat CO_2 (Fig. 5.5) was the highest for the cream of Protease P (0.34 g extract/g CO_2).

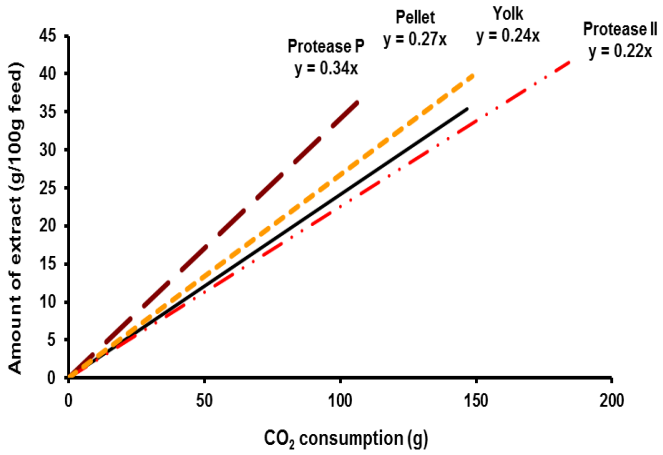

Figure 5.5. The CO_2 loading (g extract/g CO_2) or slope of initial linear portion of the extraction curve with neat CO_2 for dry yolk, pellet and creams.

Higher slope values mean easier accessibility of lipids and higher extraction rate, resulting in shorter extraction times. The slope was the lower for cream of Protease II (0.22 g extract/g CO_2), then for dry pellet and yolk. Creams, especially from Protease P, had a soft and oily texture and could be easily coated on the surface of glass beads. This could have increased the surface area and the contact between CO_2 and sample and thus facilitate CO_2 diffusion into the matrix, resulting in better accessibility and extractability of lipids. Nevertheless, CO_2

loading for dry yolk and pellet (0.24 and 0.27 g extract/g CO_2, respectively) was still higher than that for cream from Protease II (0.22 g extract/g CO_2).

5.3.4. Phospholipids content of the extracts

PL chromatograms of yolk (solvent and SC-CO_2+ethanol) and cream (SC-CO_2+ethanol) are shown in Figure 5.6. No PL could be detected in extracts from neat CO_2. Egg yolk lipids extracted with organic solvent showed two distinct peaks related to PC and PE (Fig. 5.6). However, in some extracts obtained with SC-CO_2+ethanol from dry yolk, the peak related to PE completely disappeared (Fig.5.6). Lower recovery of PE during the sample preparation and limit of quantification of the UPLC could be a few reasons why the PE peak could not be observed in the related chromatograms. However, it seems that PE solubility and therefore its recovery using SC-CO_2+ethanol is lower than that of PC. After enzymatic hydrolysis, some PC to LPC conversion happened and a small peak related to LPC appeared in the chromatograms of extracts of Protease P and Protease II treatments (Fig. 5.6).

The purity and recoveries of PC and PL based on original egg yolk (PL recovery (1) shown in Fig. 5.2.) are presented in Table 5.2. The recoveries of PC and PL increased substantially after enzymatic hydrolysis of the pellet (Table. 5.2). However, only the hydrolysis of the pellet with Protease P could improve the recovery of PC (85%) and PL (86%) significantly, compared to that from dry yolk.

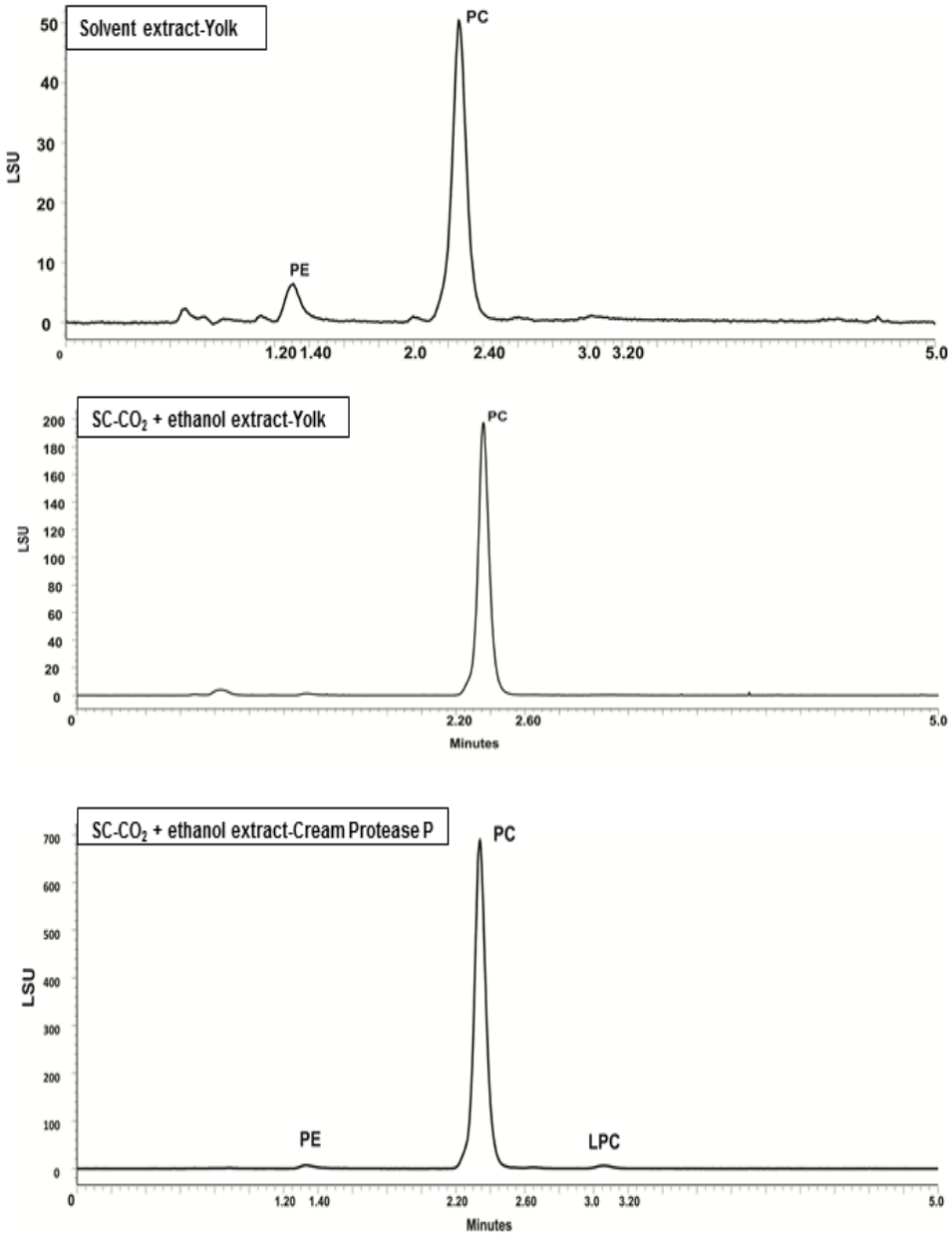

Figure 5.6. PL chromatograms (PC: Phosphatidylcholine, PE: Phosphatidyl-ethanolamine and LPC: Lysophosphatidylcholine of extracts of yolk (solvent and SC-CO_2+ethanol) and cream (SC-CO_2+ethanol).

Although the PC and PL purity slightly improved after enzymatic treatment with Protease P, the increase was not statistically significant. Proteins are not soluble in SC-CO_2 (Cape et al., 2008); however, the addition of ethanol into the SC-CO_2 stream can lead to solubilisation of small peptides, especially after yolk hydrolysis. Therefore, enzymatic treatment co-extracts peptides at increasing ethanol, which could decrease the overall purity of the extracted PL.

Interestingly, extracts from the pellet had the highest PC and PL purity at about 75 and 88%, respectively (Table 5.2.). During IgY extraction, the major part of soluble proteins are removed into the supernatant by 10-fold dilution. Therefore, the higher purity of PL extracted from the pellet compared to the yolk or creams might be due to the removal of soluble peptides and proteins, which in part could have been co-extracted in the presence of ethanol. However, the characterisation of such impurities in the SC-CO_2+ethanol extracts needs further investigation to understand the nature of impurities and therefore how to reduce them in the final product.

Table 5.2. The recovery and purity of PC and PL (PC+PE+LPC) of different treatments (yolk-based, PL recover (1) according to Fig. 5.2.). [1,*]

Treatment	PC[1]		PL[1]	
	Recovery	Purity	Recovery	Purity
Dry yolk	47±11	47±20	49±9	45±14
Dry pellet	40±10	75±12	25±6	89±11[*]
Cream Protease II	72±18	42±10	75±25	51±15
Cream Protease P	85±6[*]	57±10	86±6[*]	67±12

[1] Mean ± standard deviation based on triplicate extractions, except for pellet, which was in duplicates.
[*]Means with (*) are significantly different from dry yolk ($p < 0.05$).

A comparison between the treatments showed that the recovery of total PL was only slightly higher than that for PC, except for the pellet sample where the PC recovery was higher than total PL. This could be reflected in the fact that PE, as the second largest PL class in egg yolk, shows quite lower solubility than PC in SC-CO_2+ ethanol. PL solubility in SC-CO_2+ethanol has been suggested to be as follows: PC> SM>PE>LPC>CL>PS (Catchpole et al. 2009). Also, at atmospheric pressure PC solubility in ethanol is substantially higher than that of PE which could explain why the overall recovery of total PL by SC-CO_2+ethanol was not much higher than that of PC recovery. In the study by Dunford and Temelli (1995) to extract PL from canola, the yield of PC obtained with SC-CO_2+ethanol extraction was higher than that for total PL. According to Dunford and Temelli (1995), higher solubility of PC in ethanol compared to other PL classes resulted in the higher recovery of PC than total PL. Using the solubility differences of PL classes in ethanol, Montanari et al. (1996) investigated selective fractionation and isolation of PL classes from soybeans. Therefore, although enzymatic hydrolysis could to some extent improve PE extractability, lower solubility of PE in SC-CO_2+ethanol was still a limiting factor.

The recovery of PC was calculated based on the feed material for the extraction (PL recovery (2) shown in Fig.5.2.) is represented in Figure 5.7. As expected, the pellet treated with Protease P and Lipase AY30 had the most positive effect on improving the extractability and the recovery of PC (94% vs. 46.8% and 39.7% for the dry egg yolk and the pellet, respectively). Several factors could be involved for the higher recovery of PC from cream. As already

explained, the reduction of proteinous compounds in cream, smaller peptide fractions in the cream and changes in the lipid structure as well as the structure of yolk could have increased the extent of CO_2 diffusion within the matrix. Meanwhile, the apoproteins hydrolysis may have depended on the non-covalent bonds between apoproteins and PL, making the PL more accessible and easier to solubilize by SC-CO_2+ethanol.

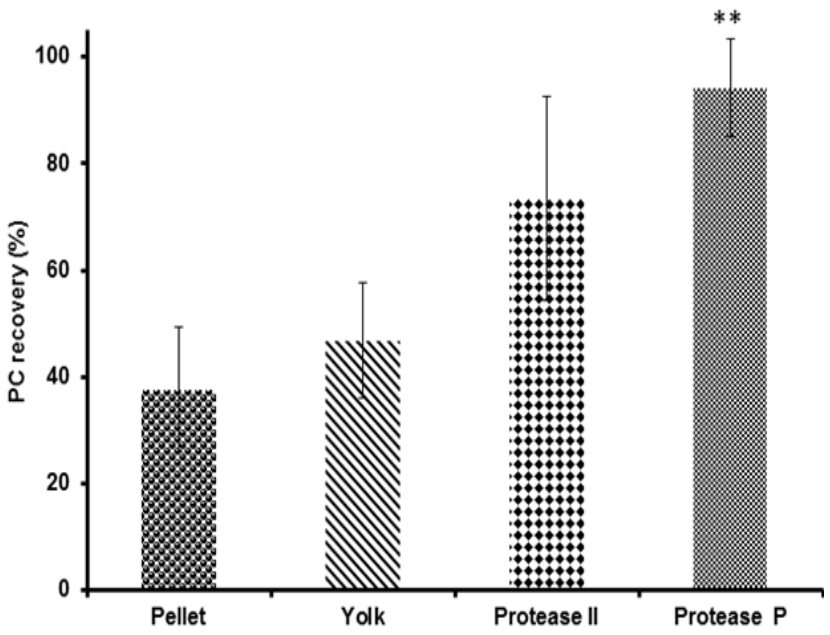

Figure 5.7. PC recovery calculation based on the feed material (based on PL recovery (2) shown in Fig. 5.2) during the SC-CO_2+ethanol extraction step based on the amount of PC in the feed material used for extraction (** indicates significant difference at $P < 0.01$ compared to yolk).

5.4. Conclusions

Our results demonstrated that enzymatic treatment could improve the extractability of PL using SC-CO_2+ethanol after treatment of the pellet with

proteolytic and lipolytic enzymes. However, not all enzymes seem to perform at the same level. Protease P treatment in combination with LipaseAY30 resulted in the recovery of over 85% of PC from yolk. The recovery of PC based on the cream used for SC-CO_2+ethanol was about 94%, while for dry yolk or pellet, PC recovery was about 47% and 39%, respectively. This showed that the structural changes of the pellet after enzymatic treatment had a great impact on the extractability of PC from yolk. PL and PC extracted from dry pellet showed higher purity than that extracted from cream. The PC purity of extracts obtained from cream of Protease P was 57.4%, which was only slightly higher than that of the egg yolk extracts (46.8%). This study also suggested that even though hydrolysing the pellet or yolk could increase the extractability of total PL, however, the recovery of specific classes of PL is another important consideration.

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6. Moisture impact on extractability of phospholipids from egg yolk after enzymatic hydrolysis[4]

6.1. Introduction

Moisture content of the feed material used for supercritical carbon dioxide (SC-CO_2) extraction has a significant effect on the extractability of solutes present in the matrix. The high moisture content can hinder the extraction kinetics by acting as a barrier for SC-CO_2 to diffuse into the matrix and can also result in possible pH alteration of the extraction environment due to CO_2 solubilisation and formation of carbonic acid. On the other hand, the presence of a small amount of moisture in biological matrices may decrease the affinity of oil for the matrix, perform as a co-solvent and induce selective extraction of certain compounds (Dunford et al. 1997a). Therefore, before their use as feed for SC-CO_2 extraction, fresh biological materials are usually dried to low levels of moisture content.

Egg yolk is a fluid emulsion with about 50% moisture (Hatta et al. 1990; Anton 2007). Due to its high moisture content, strong emulsion structure and the position of PL in its structure, i.e. their bonding to apoproteins, the diffusivity of CO_2 into the fresh egg yolk and extractability of PC can be very low (Burely and Vadehra 1989). Therefore, similar to other biological materials, egg yolk dehydration is a prerequisite to de-oiling and PL extraction using SC-CO_2. Regarding PL extraction from egg yolk using SC-CO_2 technique, despite all

[4] A version of this chapter is to be submitted to Journal of Food Science for publication.

achievements in terms of the know-how and development of this technology, the low recovery of PC, even in the presence of the ethanol as the co-solvent, has been a great barrier for scale up purposes (Shah et al. 2004; Aro et al. 2009).

On the other hand, the extraction and fractionation of PL from egg yolk using conventional organic solvents is a complicated process, which involves the use of a large amount of hazardous organic solvents, specifically during neutral lipids removal (de-oiling process) (Schneider 1989; Nielsen and Shukla 2004; Palacios and Wang 2005a,b; Gadkowski et al. 2012). Therefore, a new processing approach that could improve the recovery of high valued PC at a satisfactory level using a "green" technology like SC-CO_2 extraction would be beneficial.

The pretreatment of the pellet by enzymatic hydrolysis prior to SC-CO_2 extraction could be a worthwhile approach to increase the extractability of PC from yolk as demonstrated in Chapter 5. However, enzymatic hydrolysis followed by a dehydration step prior to SC-CO_2 extraction of PC can be very costly for scale up purposes. Therefore, it is important to establish the extent of drying necessary to achieve efficient extraction of PC from fresh hydrolysate.

Therefore, the objectives of this study were: a) to investigate the extractability of PC and total PL from fresh egg yolk with intact structure and high moisture content compared to fresh pellet hydrolysate as well as intermediate moisture and dry ones, as well as dry yolk; b) to determine the effect of moisture content on the recovery and selective extraction of the two major PL classes in egg yolk (i.e. PC and PE) using SC-CO_2+ethanol.

6.2. Materials and Methods

6.2.1. Egg yolk pellet preparation and enzymatic treatment

Pellet was prepared as described at Section 3.2.1. For enzymatic treatments, the fresh pellet was hydrolysed by a combination of Lipase AY 30 and Protease P as described in previous Section 4.2.2 and 4.2.5. Cream phase was separated from the subnatant liquid by puncturing the bottom of the tubes and removing the liquid part. Cream part was collected, divided into 50 mL tubes and stored at -70^{o}C.

6.2.3. Proximate analyses

Proximate analyses of samples were performed as described at 3.2.3.1.

6.2.4. SC-CO_2 extraction process

For SC-CO_2 extraction, the following treatments were as used feed material: fresh egg yolk, dry yolk, and creams at different moisture levels: fresh (45% moisture), partially dried (20% moisture) and freeze-dried cream. A general scheme of the overall experimental procedure for the whole process and SC-CO_2 extraction was given in Figure 6.1. To obtain an intermediate moisture feed (20%), cream was frozen at -70 ^{o}C and then freeze dried to target level of moisture content (Labconoco, model 7806020, Kansas, MO, USA). The laboratory scale supercritical fluid extraction system and the protocols described in Section 5.2.4. were used for the extraction process. SC-CO_2 extraction was performed in two steps, where ethanol was added as co-solvent into SC-CO_2 in the second step. Extracts were collected in glass tubes and dried at 50 ^{o}C under a gentle nitrogen stream until its weight reached a constant level and recorded.

The recovery of PC and total PL in the SC-CO_2 extracts was calculated as described in Section 5.2.5.2. i.e. recovery based on SC-CO_2 extraction feed (PL recovery (1)) and yolk-based recovery (PL recovery (2)). However, during the material balance calculation the moisture content difference of samples were taken into account.

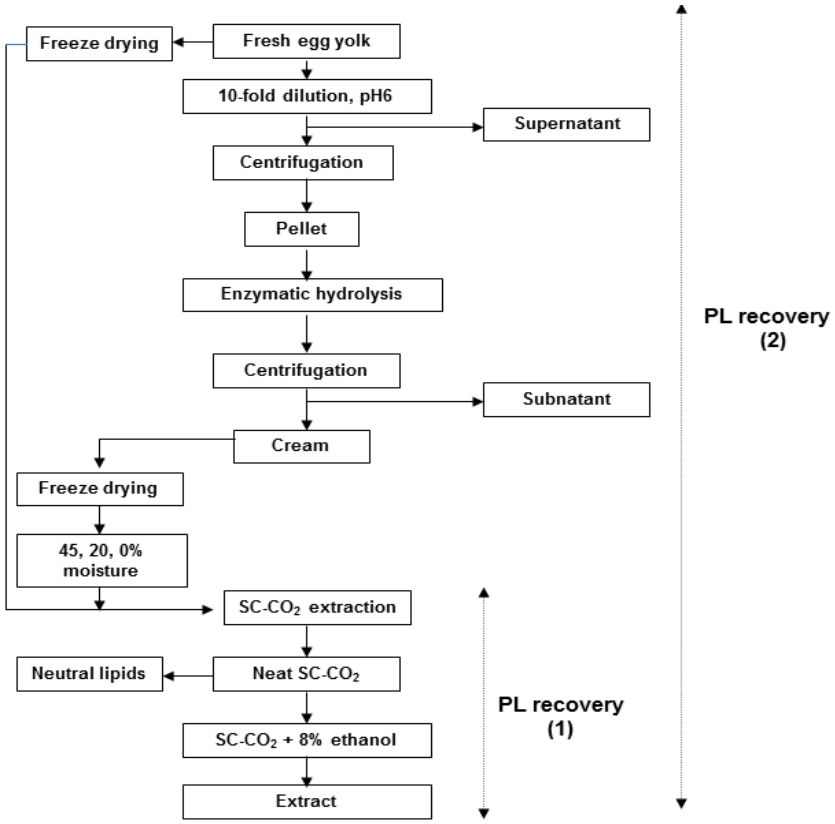

Figure 6.1. Flow diagram of sample preparation and SC-CO_2 extraction protocols.

6.2.5. Phospholipids analysis

Sample preparations for PL quantification were according to the procedures described in previous chapters (Chapters 4.2.61 and 4.2.6.2).

6.2.6. Particle size distribution of pellet hydrolysates

The volume weighted mean diameter (*d4,3*) of prepared cream was measured by laser light scattering particle size analyser (Mastersizer 2000 S, Malvern). About 0.3 g of sample was diluted in 10 mL 0.01% SDS solution and vortexed. The refractive index for water as the dispersant was 1.33 and for lipid was set as 1.6 at 20 ^{o}C. Measurements were performed in duplicates for two independent treatments.

6.2.7. Optical microscopy

The fresh cream phase was diluted in water; a small amount was smeared on a microscopy slide and covered with a cover slip. One drop of immersion oil was put on the cover slide, which was then visualized under optical microscopy (Zeiss-Axiocam ERc 5s, Goettingen, Germany) with 100X objective.

6.2.8. Cryo-Scanning Electron Microscopy

Small amounts of fresh egg yolk, fresh cream and dry cream were taken and super-cooled in liquid nitrogen and inserted into the cryo-preparation system (Emitech K1250, UK) where the free water in the samples was sublimed. Then, the surface was coated with gold in deep frozen state and were transferred into SEM (JEOL 6301F field emission SEM, Japan).

6.2.9. Statistical analyses

Treatments for statistical analyses were fresh yolk, dry yolk, and the creams obtained from Protease P and Lipase AY30 treatment dried to different moisture levels (full-freeze dried, 20% and 45%). All SC-CO_2 extractions were performed at least in duplicates. Results of PL recovery and purity were subjected

to general linear model, one-way analysis of variance (ANOVA) using GRAPHPAD PRISM (Graph pad software, San Diego, CA, USA) at a confidence interval of 95% ($p < 0.05$). Means were compared to determine significant differences at $p < 0.05$ by using the Tukey's HSD test.

6.3. Results and discussions

6.3.1. Composition of fresh cream and yolk

The lipid content and PL composition of yolk and the cream have been shown in Table 6.1. Yolk had about 65.3% lipids on dry matter basis, whereas that for the cream was 84.3%. PC and PE were the two major PL classes detected and quantified in the yolk. PC was the major component for both egg yolk and cream comprising 15% and 16.1% of dry yolk and dry cream weight, corresponding to about 72.8% and 67% of total PL content, respectively. Of the three batches of enzymatic treatments conducted, one batch had LPC in detectable quantity. LPC is one of the PL groups in egg yolk, available in a small amount (Rossi 2007). Due to the detection limit of the current analytical method employed, LPC could not be detected and quantified in the chromatograms of the yolk samples.

Table 6.1. Total lipid and major PL contents (g/100g dry matter) of yolk (n=2) and cream (n=3) used as feed material for SC-CO_2 extraction.

Sample	Total Lipid	PC	PE	LPC
Yolk	65.3±1.8	15±1.7	5.6±1.4	N.D.
Cream	84.3±3.5	16.1±1.5	6.2±2.6	1.7±1.9

N.D. not detected

6.3.2. SC-CO_2 extraction curves

Cumulative extraction yield curves for fresh yolk, dry yolk as well as fresh (45%), intermediate dry (20% moisture) and freeze-dried creams were plotted as a function of the amount of CO_2 used over the two steps of extraction, involving neat CO_2 followed by CO_2+ethanol (Fig. 6.2).

Extracts were dried completely under a gentle stream of nitrogen until constant weight was reached before recording their weight. Therefore, the amount of water extracted from wet samples was not included and quantified in this study. Overall extraction curves showed the same trend for all treatments, including a gradual increase in the mass of extract, establishing the typical initial linear region with easily accessible lipids being solubilized in the neat CO_2, then reaching the plateau where extraction was limited by diffusion, followed by another increase in the cumulative weight of extract due to solubilisation of polar compounds in ethanol+CO_2 stream and then reducing a second plateau. As shown in Figure 6.2, the time required (or CO_2 consumption) to obtain the full curve for fresh egg yolk was much longer compared to that of dry yolk or cream, demonstrating that the strong emulsion structure of intact yolk was a barrier for CO_2 diffusion and solubilisation of lipids.

Considering only the total mass of feed material placed in the extractor, there was a substantial increase in the total amount of extract with a decrease in the moisture content of the feed material. On the other hand, cumulative extraction yield based on dry mass of feed material (dry basis calculation, Fig. 6.2b) was higher for the cream with 45% moisture where 82% of total dry matter

was extracted during the two-step extraction. This yield was even higher than that of dry cream (73%). The lowest cumulative yield was for the fresh yolk where about 44% of its dry matter could be extracted in the two-step extraction.

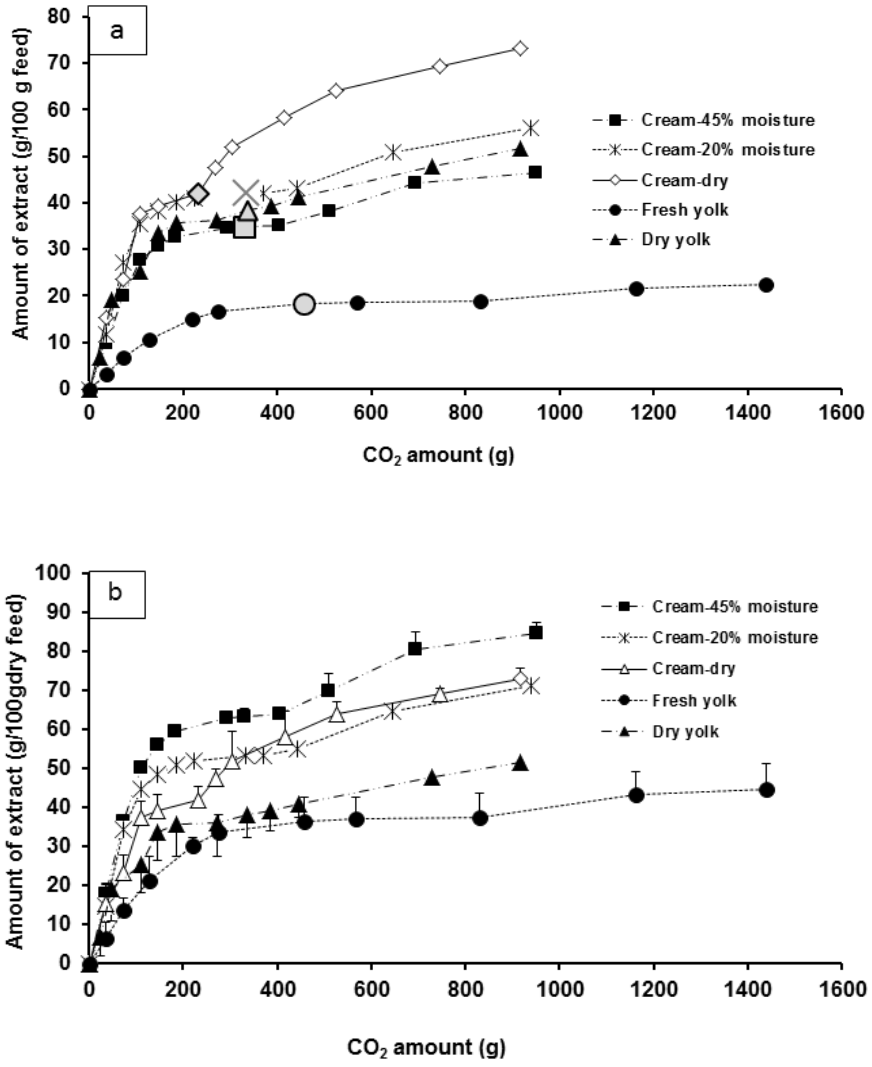

Figure 6.2. a) Total extract yield of different treatments using the two-step SC-CO_2 extraction process based on original mass of feed. Larger gray symbols show the start of ethanol injection. b) Total extract yield of the same treatments calculated based on original dry mass in the feed material. Data for dry yolk and dry cream are from Chapter 5 for comparison purposes.

The slope of the initial linear part of the extraction curves, also called CO_2 loading (g extract/g CO_2), obtained during the first step of extraction (Fig. 6.2a) with neat CO_2 (Fig. 6.3) was higher for the creams. Higher slope values mean easier accessibility of lipids and higher extraction rate, resulting in shorter times required for the extraction. The CO_2 loading was overall higher for the creams, and it decreased with an increase in the moisture content of the cream. The highest CO_2 loading values were for the dry and intermediate dry (20% moisture) curves, while fresh yolk had the lowest value. The CO_2 loading for fresh cream (45% moisture) was still higher than that for dry yolk. Dunford et al. (1998) showed that during the extraction of oil from mackerel fish muscle, oil solubility significantly decreased at moisture contents above 45%. However, it seems that enzymatic digestion improved the total extractability of material from egg yolk even in the presence of high moisture content (45%).

Therefore, from the current study, it can be concluded that the structural changes due to the degradation of larger proteins into smaller peptide fractions and their partial removal showed higher impact than that of water on accessibility and solubility of lipids and the overall extractability of compounds in both steps of extraction.

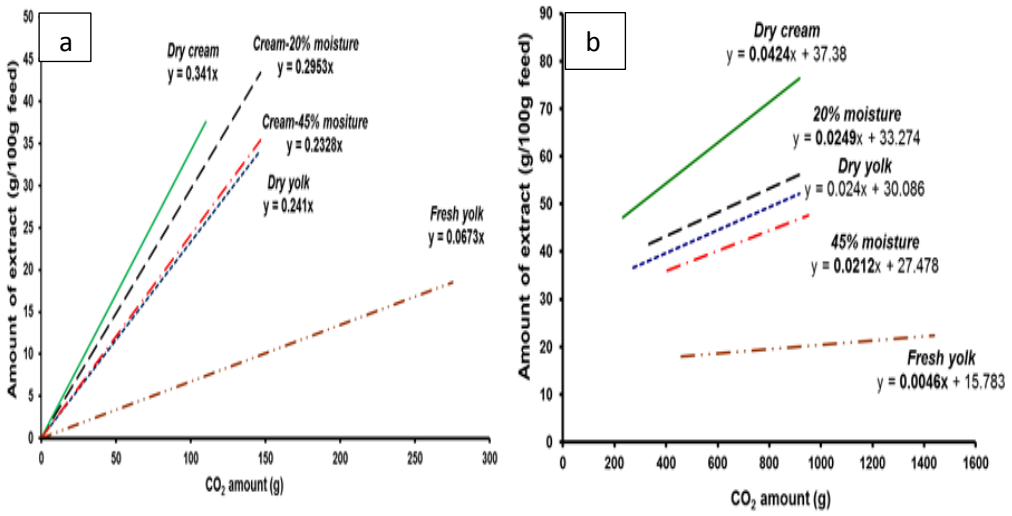

Figure 6.3. CO_2 loading (g extract/g CO_2) or slope of the initial linear portion of the extraction curves given in Figure 6.2 during the (a) first and (b) second steps of extraction with neat CO_2 and CO_2+ethanol, respectively.

6.3. Phospholipids content

6.3.1. Recovery based on extraction feed material

PL recovery was first calculated using the amount of PL available in the feed material and the amount that was recovered directly from the feed using SC-CO_2 and SC-CO_2+ethanol (Fig. 6.1, PL recovery (1)). Therefore, the amount of PC and PE were measured in the feed materials using solvent extraction and compared to the amount of PC or PE extracted by SC-CO_2+ethanol. This recovery calculation indicates the direct effects of structural modification and moisture content of feed material on PL extractability as well as PC and PE solubility behaviour in SC-CO_2+ethanol in the presence of different levels of water. Table 6.2 shows the recoveries of PC and PE from creams with different levels of moisture content compared to those for dry yolk and fresh yolk.

Overall, the highest recoveries of PC and PE were for creams where over 90% of PC could be extracted from the cream samples (Table 6.2.). As already discussed, the presence of high amount of moisture in the feed material can hinder CO_2 contact with potential solutes and decrease the extraction yield and recovery of target compounds. However, it seemed that after hydrolysis of the samples the impact of moisture on PL extractability was reduced.

Overall, the presence of high moisture content (45%) did not significantly affect the extractability of PC and PE from creams. Considering the fact that both fresh yolk and cream had almost the same level of moisture; the significantly lower recovery of both PC and PE for fresh yolk, indicates that structural changes of egg yolk after enzymatic digestion had a positive effect on the extractability of PC and PE. The structure of biomaterials used for SC-CO_2 extraction has been reported to have a significant effect on the extractability of target compounds. Mechanical treatments like crushing seeds before SC-CO_2 extraction can increase exposure of oil and CO_2 and increase the extractability of oil (Stahl et al., 1980; Snyder et al., 1984).

Froning et al. (1998) reported that both particle size and moisture content had a great impact on extractability of neutral lipids and cholesterol since more lipids could be removed using larger particles. Aro et al. (2009) also concluded that extraction yield of PC and PE was lower for fine powder dry yolk compared to the ones with granule structure, due to channelling in egg powders with small particle size. Froning et al. (1998) and Dunford et al. (1997) recommended 7% and 10% moisture presence in dry yolk and fish muscle, respectively to enhance

the extractability of neutral lipids and the presence of higher ratios of water decreased the extractability of lipids. However, to the best of our knowledge, no study has been conducted to investigate the effect of enzymatic treatment on PL extractability from fresh material. Dry yolk lacks the emulsion structure and is in solid particle form. Creams regardless of their moisture content had soft structure and could easily spread and cover glass beads with a thin coating, while fresh yolk had a viscous liquid like structure which could easily flow.

Table 6.2. The recovery of PC and PE (based on feed used for SC-CO_2 extraction) and purity of PC and PL for different treatments.

Treatment	PC-Recovery[1]	PE-Recovery[1]	PC-Purity[1]	PL-Purity[1]
Dry-cream	94±9 [a]	40±12[a]	57±10[a]	67±12[a]
Cream-20% moisture	106±4[a]	44±4[a]	84±2[b]	103±0.9[b]
Cream-45% moisture	90±9[a]	26±2[ac]	61±2[a]	65.4±4[a]
Dry yolk	47±11[b]	2.3±4[b]	47±20[a]	45±14[a]
Fresh yolk	46±1[b]	15±0.5[bc]	88±14[b]	93.4±6[b]

[a-c] Numbers in the same column with the common letter superscripts are not significantly different ($p > 0.05$). Data for dry cream and dry yolk were taken from Chapter 5 for comparison purposes.
[1]Mean±standard deviation based on duplicate extractions, except for dry cream, which was in triplicates.

Comparison between particle size distribution of intact egg yolk and the fresh cream in Fig. 6.4 showed that egg yolk had a monomodal particle size distribution with an average particle size of about 2 μm. After enzymatic hydrolysis, larger particles (oil droplets) with an average size of 53 μm, and a particle size distribution ranging between 100-1000 μm were formed (Fig. 6.4a).

Larger oil droplet size meant that cream emulsion was less stable. The creams in dry or fresh form could be easily coated on the surface of glass beads,

which could significantly increase the contact between the solutes and solvent and the diffusion rate. Cream after hydrolysis was still an emulsion as shown in Figure 6.4b. After enzymatic digestion, proteins and large apoproteins from egg yolk were hydrolysed into smaller peptides. These peptides had less emulsifying activity, since they could not cover the oil droplet as efficiently as large and flexible proteins like apoproteins. Therefore, peptides formed a thinner interfacial layer around the oil droplets, resulting in larger oil droplets which were prone to external forces including high pressure and SC-CO_2 diffusion.

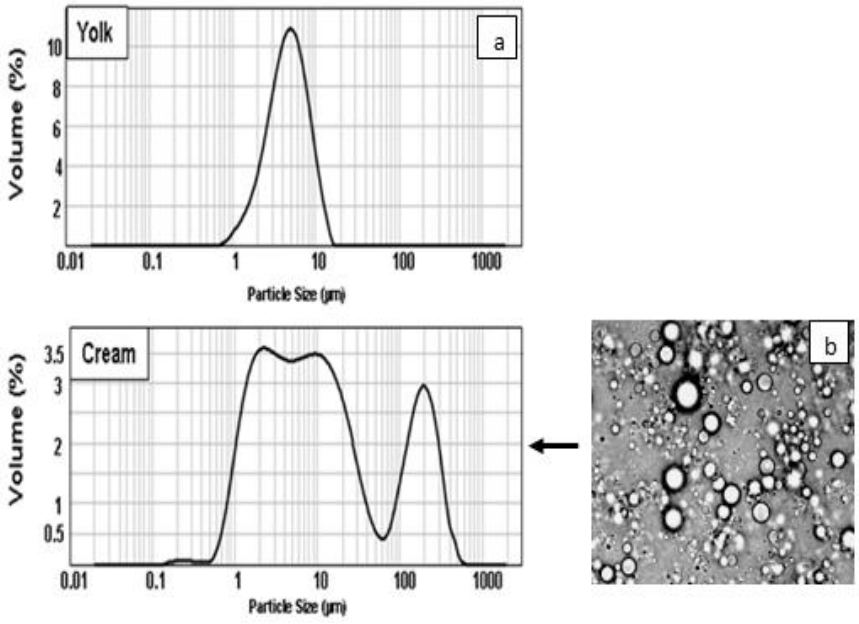

Figure 6.4. a) Particle size distribution of egg yolk and cream (45% moisture) b) Light microscopy image of cream showing oil droplets.

SEM images also showed the significant difference between creams and fresh yolk structures (Fig. 6.5). Fresh egg yolk had an obvious network of protein and large spherical particles, the so called "yolk droplets" where lipids were

trapped in those droplet structures. Creams did not have any distinct morphology. It seemed that hydrolysed lipid and hydrolysed proteins were mixed homogenously and no structure or network could be observed. The fresh cream had darker colour and "rough" structure, probably due the fact that it was not subjected to the extra freeze drying step like the dry cream. Therefore, fresh cream might contain a small amount of water and a softer structure even after sublimation for SEM, which affected the colour and appearance of the cream.

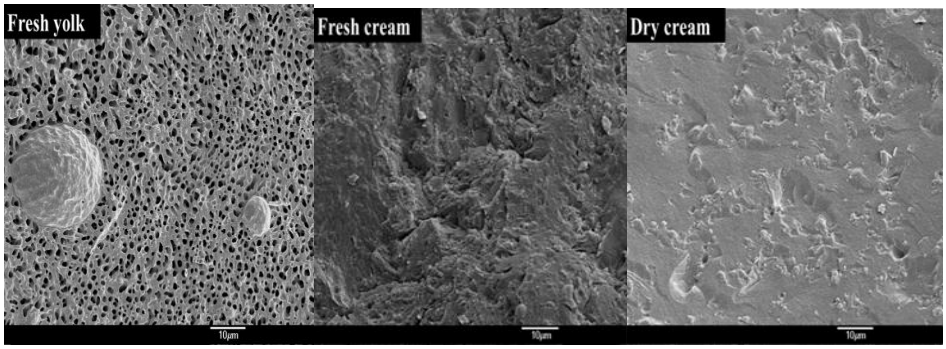

Figure 6.5. Cryo-SEM images of fresh egg yolk, fresh cream and dry cream structures (bar length: 10 µm).

Water and ethanol are the only GRAS (generally regarded as safe) solvents that can be added to SC-CO_2 as co-solvents when targeting food applications (Temelli 2009; Catchpole et al. 2009). Therefore, being a polar solvent, the water available in creams could also act as a co-solvent along with ethanol to increase the solubility of PL in SC-CO_2. Comparison of PE recoveries obtained from dry yolk and fresh yolk (2 vs. 15%) (Table 6.2), showed that water had some co-solvent effect and improved the extractability of PE. Overall, in

combination with structural changes after hydrolysis and in the presence of 20% water, maximum 43% of PE in the cream was extracted.

Co-solvents in SC-CO_2 contribute to the extraction of solutes by increasing the density of solvent. The entrainer effect refers to a simultaneous increase in solubility and selectivity of solvent, when there are some specific intermolecular interactions such as H-bonding between the functional groups of solutes and co-solvent. Therefore, the solvent may show some selectivity towards specific compounds (Guclu-Ustundag and Temelli 2005). The highest ($p < 0.05$) purity of PC in the extracts obtained from fresh yolk and cream with 20% moisture content was 84% and 88%, respectively, which may suggest that water present in specific ratios may play an entrainer role towards PC (Table 6.2). Water also could be considered an entrainer for PE when PE recoveries from dry yolk and fresh yolk (2 vs. 15%) are compared.

The highest PL (PC+PE) purity was again for the cream with 20% moisture content where the PL purity was 100%, while for fresh yolk it was about 93%. However, at higher concentration of water for cream (45%), the purity of PC and overall PL dropped to 61% and 65%, respectively. It was already shown in Figure 6.2b that the total extraction yield based on dry matter of fresh cream (45%) was the highest among all treatments. Assuming that all water was completely removed from the extracts before recording their weights; lower purity of PL in the extracts from fresh cream could be due to the presence of some other lipids as well as some polar peptides co-extracted with PL. Weaker structure of cream and the presence of high amount of water and ethanol as co-solvents may

increase the chance for the extraction of peptides, resulting in lower PL purity. Also, CO_2 solubilisation in water leads to acidic pH due to carbonic acid production. This may impact the solubility of peptides and other compounds, for example through approaching the isoelectric point of some peptides, or through destabilisation of some of the existing interactions. Therefore, the presence and function of water during SC-CO_2 extraction along with ethanol is quite complicated and requires further investigation.

This study proved that the modification of the structure of yolk can tremendously improve the extractability of PC and PE. At the same time, the selectivity of SC-CO_2 and co-solvents employed should not be neglected. As demonstrated, the recovery of PE in SC-CO_2, even in the presence of polar co-solvents such as ethanol and water is not as high as that of PC. Meanwhile, the presence of water as a polar solvent itself and its impact through altering pH of the extraction environment may enhance the selectivity for PL over other compounds, resulting in an extract with a very high PL purity. It can be concluded that a combination of enzymatic treatment and entrainer effect of water and ethanol could improve the extractability and purity of PC and PE (Table 6.2).

6.3.2. Yolk-based recovery

The recovery of PL in the final extract was also calculated considering the original amount of egg yolk mass required for producing a specific amount of cream, representing the recovery over the whole process (Fig. 6.1, PL recovery 2). Considering the fact that some lipids may be lost during the process, especially during the 10-fold dilution for IgY separation and the pellet preparation and

hydrolysis, this calculation provided useful information on the overall feasibility of the whole process in terms of the final recovery and purity of PC or PL based on the amounts present in the original egg yolk. The PC and total PL (PC+PE) recoveries for SC-CO_2+ethanol extracts of dry yolk were 46.7% and 48.8%, respectively (Fig. 6.6).

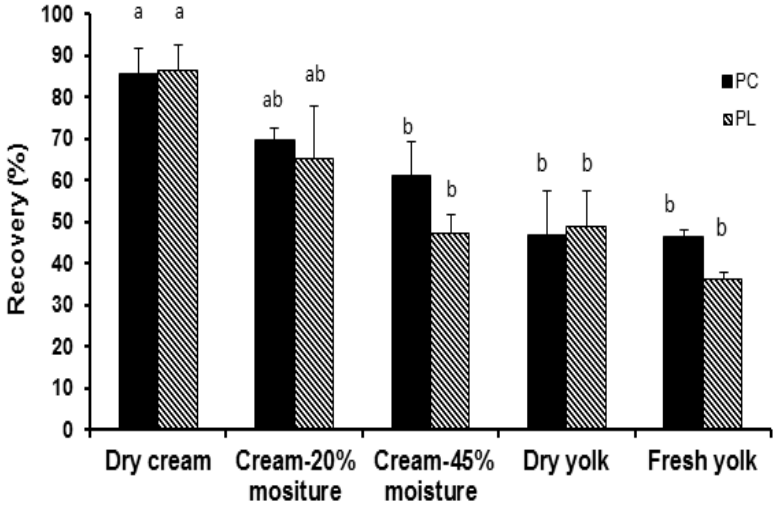

Figure 6.6. The recovery of PC and total PL (PC+PE+LPC) of different treatments based on the original yolk (n=2). Bars with the same letters in each category common letters in the same columns are not statistically different ($p < 0.05$).

The highest recoveries for PC and total PL (PC+PE+LPC) were for dry cream in which over 85.5% of PC and 86.4% of total PL from the initial mass of yolk were recovered in the final extract ($p < 0.01$). The recoveries of PC and PL were also significantly higher for the cream with intermediate moisture (~69.8% at $p < 0.05$). The PC recovery for cream with 45% moisture was higher than that of dry yolk (61.2% vs. 46.8%), but it was not statistically significant. Similar recoveries for dry and fresh yolk samples do not mean that fresh egg yolk would

be a suitable material for direct PL extraction. As demonstrated in Figure 6.2, to extract the same amount of PC from fresh yolk considerably longer extraction time and ethanol consumption was required.

6.4. Conclusions

Dry yolk, fresh yolk and its hydrolysed fractions (creams), at different levels of moisture content (0, 20, 45% w/w), were subjected to a two-step SC-CO_2 extraction process. In the first step, neutral lipids were removed from samples using neat CO_2 and in the second step, SC-CO_2+ethanol was used for PL extraction. The cream fraction showed significantly higher recoveries of PC and PE at dry and intermediate level moisture compared to dry yolk or fresh one. With intermediate moisture content of 20% (w/w) of cream, an extract with a high purity of PC (84%) and total PL (103%) was recovered from cream. Overall, it seemed that SC-CO_2+ethanol showed more selectivity towards PC. This study showed that enzymatic hydrolysis of egg yolk is a promising approach to recover high-valued PC using SC-CO_2 technique. Meanwhile, the amount of water extracted during the process was not determined in this study. The exact determination of water amount to be co-extracted in the final product is an important aspect, which requires further investigation.

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7. Interaction between egg yolk and ionic polysaccharides: a potential approach for isolation of low density lipoproteins[5]

7.1. Introduction

Low density lipoproteins (LDL), accounting for about 70% of egg yolk dry matter and containing about 90% of egg yolk lipids, are small spherical particles of 16-70 nm diameter (Martinet et al., 2003). Due to their high content of PL and unique nanostructure, LDL from egg yolk have great potential as carrier vehicles for delivery systems and high value choline-rich PL recovery. A tedious ultracentrifugation method has been used for LDL separation, but the LDL yield is low and the protocol is time consuming (Moussa et al., 2002). Another method, consisting of egg yolk fractionation by dilution, ammonium sulphate precipitation and dialysis was also developed to isolate LDL by Moussa et al. (2002). Although the LDL yield was improved in this method up to 67%, a dialysis step was required, which may not be suitable for large scale preparation of LDL.

Polysaccharides are abundantly available natural macromolecules and their addition to food systems can improve emulsion stability, which may lead to novel products In addition, polysaccharides have shown potential for selective separation of proteins and lipids from complex systems such as dairy waste and egg yolk to obtain high value bioactive components and improve nutritional properties (Hatta et al., 1988,1990; Shah and Singh, 1992; Casal et al., 2006). The use of slightly anionic polysaccharides for the removal of cholesterol and PL from egg yolk has been reported previously. Hatta et al. (1988) used sodium alginate to

[5] A version of this chapter will be submitted to Carbohydrate Polymers for publication.

precipitate lipoproteins from 6-fold diluted egg yolk to obtain a lipid-free supernatant fraction for further immunoglublin Y (IgY) purification. Rojas et al. (2007) examined the impact of several factors such as dilution and ionic strength on cholesterol removal from egg yolk using high-methoxyl pectin and reported 6-fold egg yolk dilution at lower ionic strengths and pH of 9.2 to be the optimum condition for the precipitation of cholesterol from egg yolk. Hatta et al. (1990) studied the effects of λ-carrageenan and xanthan gum on the purification of IgY from 6-fold diluted egg yolk. Both polysaccharides could precipitate LDL; however, λ-carrageenan gave better results regarding the removal of LDL and improvement of overall IgY extraction yield.

The studies summarized above focused on the removal of lipids from egg yolk in order to separate IgY, where LDL were co-precipitated along with other proteins such as high density lipoproteins (HDL) and phosvitin in the pellet phase. To the best of our knowledge, no other studies have focused specifically on the separation of LDL into a distinct phase from whole egg yolk using polysaccharides. Therefore, the main goal of this study was to investigate the potential of polysaccharides for the isolation of LDL from 2-fold diluted egg yolk. The 2-fold dilution factor was selected based on the preliminary studies in this work regarding phase separation behaviour (data not reported).

The polysaccharides used in this study were xanthan gum, *ι*-carrageenan and gum arabic, which are all anionic, but with prominent differences in their chemical backbone and rheological properties. Xanthan gum is an exo-polysaccharide produced by the microorganism *Xanthamonas campestris.*

Xanthan gum can be dissolved in cold water and its solutions exhibit pseudoplastic behaviour (Phillips and Williams, 2000). Its structure consists of a linear (β 1→4) linked D-glucose backbone with trisaccharide side chains of glucuronic acid and mannose attached to the C-3 position of every other glucose unit. About 50% of all terminal mannose residues are pyruvylated (Phillips and Williams, 2000). Gum arabic, originating from plant seeds, is a complex polysaccharide consisting of sugars such as rhamnose, glucuronic acid and arabinose as well as some proportion of nitrogenous compounds, including amino acids. Gum arabic is easily soluble in water, and its solution shows Newtonian behaviour (Phillips and Williams, 2000). Carrageenans are comprised of a galactose backbone with different proportions and locations of ester sulphate groups. Carrageenans are classified as κ-, λ- and *ι*-carrageenans, based on their structural differences in terms of anhydrogalactose and ester sulphate contents. *ι*-Carrageenan, used in this study, is soluble in cold water and its viscosity at similar concentrations is much lower than that of xanthan gum (Phillips and Williams, 2000).

Based on the differences in the properties of the three selected polysaccharides, the specific objectives of this study were to understand possible interactions and following phase separation behaviour after mixing egg yolk components and the polysaccharides at different concentration and pH conditions. Treatments with indications of possibility for LDL isolation were selected for further structural and chemical analyses.

7.2. Materials and Methods

7.2.1. Materials

White shell eggs (Grade A), produced by Sparks egg farmers of Leucerne Inc. (Calgary, AB, Canada) were obtained from a local supermarket (Safeway Inc., Edmonton, AB, Canada). Xanthan gum and *ι*-carrageenan (Commercial grade, Type II) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Gum arabic was obtained from Fluka Analytical (Saint Quentin-Fallavier, France).

7.2.2. Sample preparation

Egg yolks were separated manually from white and gently rolled on Whatman paper to remove albumen. The vitelline membrane was punctured with a sharp blade and egg yolk content was collected in a beaker placed in an ice bath. Polysaccharide solutions were prepared by adding 40, 80 and 120 mg of each polysaccharide into 20 g of MilliQ water in beakers covered with paraffin film, and stirring at ambient temperature (20 ^{o}C) until the polysaccharides became completely dispersed and homogenous. Yolk/polysaccharide suspensions were prepared by mixing 20 g of fresh yolk with the polysaccharide solutions to achieve final polysaccharide concentrations of 0.2, 0.4 and 0.6% (w/w, weight of polysaccharide/weight of fresh egg yolk). To study the effect of pH on egg yolk/polysaccharide interactions, pH value was adjusted to 3, 5, 6, 8 and 10 by adding 2 M NaOH or HCl. After pH adjustment, egg yolk/polysaccharide suspensions were mixed for 1 h at room temperature using a magnetic stirrer at 500 rpm.

7.2.3. Phase separation behaviour

Yolk/polysaccharide and yolk suspensions with no polysaccharide addition were centrifuged at 6,000xg for 30 min to observe the phase separation behaviour of yolk/polysaccharide suspensions. According to Mcbee and Cotterill (1979), egg yolk can be fractionated into two distinct phases, a soluble phase called plasma and an insoluble fraction referred to as granules by 2-fold yolk dilution with water at pH 6, which is the natural pH of egg yolk. About 40 mL of suspension was transferred to 50 mL tubes and centrifuged to observe the phase separation.

To study plasma/xanthan gum phase separation behaviour, 40 mL of plasma from 2-fold diluted egg yolk was mixed with 80 mg of xanthan gum and mixed for 1 h and then centrifuged. All fractions obtained after centrifugation were carefully separated and freeze dried (Labconco, model 7806020, Kansas, MO, USA) for protein profile analyses.

7.2.4. Lipid content determination

To determine the total lipid content, samples were first freeze dried (Labconco, USA). Lipid content of the freeze-dried sample was determined according to Section 3.2.3.1.

7.2.5. Zeta potential (ζ)

Zeta potential of egg yolk/polysaccharide suspensions at different pH levels was measured using Zetasizer 2000 (Malvern Instruments, Worcestershire**,** UK). About 0.2 g of sample was diluted to a final volume of 40 mL using MilliQ water and the pH was re-adjusted to the study pH (3, 5, 6, 8 or 10) with 0.1 M

HCl or NaOH solution. Samples were stirred before measurements. Each sample was measured three times. Each measurement was an average of six readings. All measurements were conducted at 20 ^{o}C.

7.2.6. Particle size distribution

Particle size distribution was measured using a Mastersizer 2000S (Malvern Instruments, Ltd., Chicago, IL, USA). Water was used as dispersant and refractive index was adjusted to 1.33. Samples were dispersed in MilliQ water at 1200 rpm until the obscuration value reached 20-30%. Samples were measured in triplicates and each measurement was an average of three readings for 15 s. Particle size was reported as the volume weighted mean diameter *(d4,3)*.

7.2.7. Confocal laser scanning electron microscopy (CLSM)

Microstructure of selected samples was studied using Leica TCS SP5 II confocal system (Leica, Mannheim, Germany). Molecular probes Alexa Fluor® 488 Concanavalin A and Alexa Fluor® 555 C2 maleimide (Molecular Probes, Invitrogen, Eugene, OR, USA) were used for dying xanthan gum and proteins, respectively. Alexa Fluor 488 was prepared at final concentrations of 200 μg/mL in sodium bicarbonate buffer at pH 8.3, while Alexa Fluor 555 was prepared in 100 μM in phosphate buffer at pH 7. About 10 mg of sample was incubated with adding 100 μL of each solution, and incubated overnight at 5 ^{o}C. The next day, samples were smeared on microscopy slides, covered with a cover slip (no.2), and examined using immersion oil × 100 objectives. The CLSM was operated at two channels using the fluorescence mode at the excitation wavelength of 495 nm and the emission wavelength of 524 nm for xanthan gum visualization; and 555 and

565 nm excitation and emission wavelengths, respectively, for protein visualization in red, while lipids fluoresced in bright yellow or yellow-orange at this range. All images were taken at a resolution of 512×512 pixels.

7.2.8. Scanning electron microscopy (SEM)

Egg yolk samples were picked up with a wooden stick, released into 2.5% glutaraldehyde in cacodylate buffer at pH 7.5 and left standing for 2 h at ambient temperature (20 ^{o}C). Samples were rinsed with the same buffer for 15 min each for three times, and then post fixed in 1% osmium in the same buffer as above for 2 h at room temperature. After this, samples were dehydrated in a series of ethanol baths of increasing concentration from 50%, 70%, 90% to 100%, each for 10 min. Then, they were dried with a critical point dryer, sputter coated with gold and then mounted onto a stub. The samples were examined using a scanning electron microscope (Hitachi S-2500, Tokyo, Japan).

7.2.9. Protein profile

The procedure for SDS-PAGE was similar to the method described in the Section 3.2.3.2. However, 4–12% Criterion™ XT Bis-Tris precast polyacrylamide gels in a Criterion Cell attached to PowerPac Basic Power (Bio-Rad Laboratories, Inc., Hercules, CA, USA) were utilized in this study and 1X MOPS buffer (Bio-Rad) was used as running buffer.

7.2.10. "Pure" LDL preparation

LDL, used as control in this study, was prepared in laboratory scale according to Moussa et al. (2002). Briefly, ammonium sulphate was added to plasma obtained from 2-fold diluted egg yolk and stirred for 1 h at 4 ^{o}C. Then, it

was centrifuged at 10,000×g, the supernatant was collected and dialyzed against distilled water at least for 6 h. Aggregated LDL were collected for the experiments.

7.3. Results and discussion

7.3.1. Effects of polysaccharide type and its concentration on particle size of egg yolk

The effect of polysaccharide concentration on the particle size of egg yolk/polysaccharide suspensions was studied at the natural pH of egg yolk (pH 6). Mastersizer results (Fig. 7.1) showed that the average particle size of egg yolk suspension without any polysaccharide addition was about 10 μm. The average particle size of yolk suspensions was not affected by the addition of gum arabic, while it increased the most by the addition of *ι*-carrageenan and to a lesser extent by the addition of xanthan gum. The maximum increase in particle size was at 0.4% of polysaccharide addition (580 μm and 108 μm for *ι*-carrageenan and xanthan gum, respectively) but it decreased at the polysaccharide concentration of 0.6% (489 μm and 66 μm).

The nature of interactions between two biopolymers such as proteins and polysaccharides can be electrostatic, hydrogen bonding, hydrophobic attractions or steric repulsion. The types of interaction are dependent on the native structure of biopolymers but can be modified drastically by environmental factors such as pH and ionic strength (McClements, 2006). Electrostatic interactions have been suggested as the most common forces for the complex formation or phase formation of biopolymer mixtures (Samant et al., 1993). The decrease in particle

size at 0.6% concentration was probably due to higher viscosity of polysaccharide solution, which makes the interactions between yolk/polysaccharides less favourable. Smith et al. (1995) and Rojas et al. (2007) also observed that during cholesterol removal from egg yolk, higher dilution factors increased the chance for complex formation between applied polysaccharides and yolk.

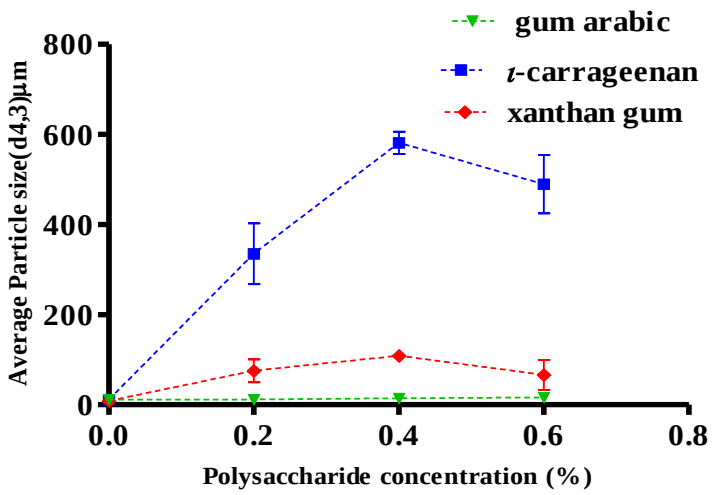

Figure 7.1. Effect of polysaccharide concentrations on volume weighted mean diameter of egg yolk suspensions at pH 6.

7.3.2. Effect of pH on egg yolk/polysaccharide suspensions at 0.4% concentration

7.3.2.1. Zeta potential (ζ)

Since the particle size of yolk suspensions was the most affected at a concentration of 0.4% of *ι*-carrageenan or xanthan gum, hereafter, all tests were performed at 0.4% polysaccharide concentration, unless otherwise indicated. To understand at which pH values the nature of interactions between yolk and

polysaccharides is electrostatic attraction or if other types of forces are involved, particle charge of LDL, yolk and yolk/polysaccharide suspensions was examined at different pH values. First, zeta potential of LDL was examined at different pH levels because over 70% of egg yolk dry matter is LDL and understanding of LDL charge at different pH conditions could be useful in explaining the nature of interactions between egg yolk and polysaccharides. Zeta potential results for "pure" LDL extracted from egg yolk showed that LDL had a slightly positive charge at pH 6 and its isoelectric point (ζ=0) was between pH 6 and 7 (Fig. 7.2).

Zeta potential for egg yolk decreased with increasing pH levels. Zeta potential of egg yolk was positive at pH 3 and 5 (35.5 and 10.4 mV, respectively), while it was negative at pH 6 (-9.58 mV) and above (Fig.7.3). Thus, isoelectric point of the yolk was between pH 5-6, close to pH 6 which is lower than the isoelectric point of LDL, the major component of yolk. The presence of other components such as soluble proteins and highly negatively charged proteins like phosvitin with a broad range of isoelectric points slightly shifted the isoelectric point of the intact yolk compared to that of LDL. Egg yolk was positively charged at pH 5. After *ι*-carrageenan or xanthan gum addition, total net charge of the egg yolk started to become more negative at pH 5 and above, but it was not affected by gum arabic addition. At alkali pH 10, *ι*-carrageenan/yolk suspension was the most negatively charged (-60 mV) of all treatments, while xanthan gum/yolk suspension had a net charge of -40 mV.

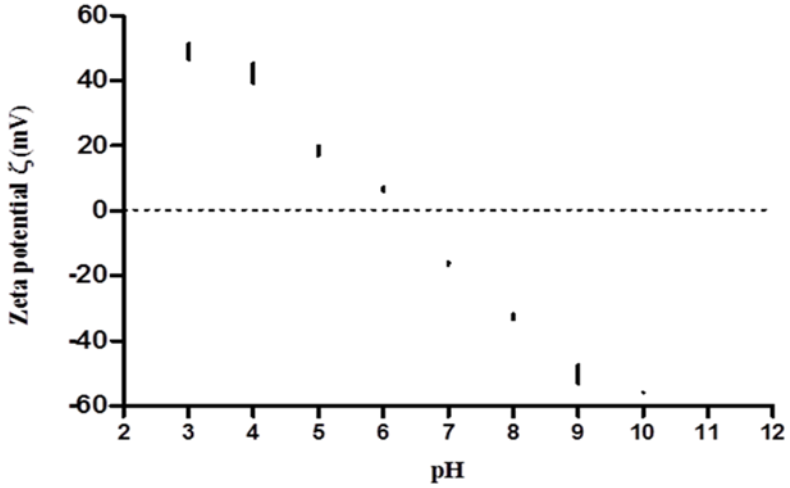

Figure 7.2. Zeta potential (ζ) of "pure" LDL extracted from egg yolk as a function of pH (n=3).

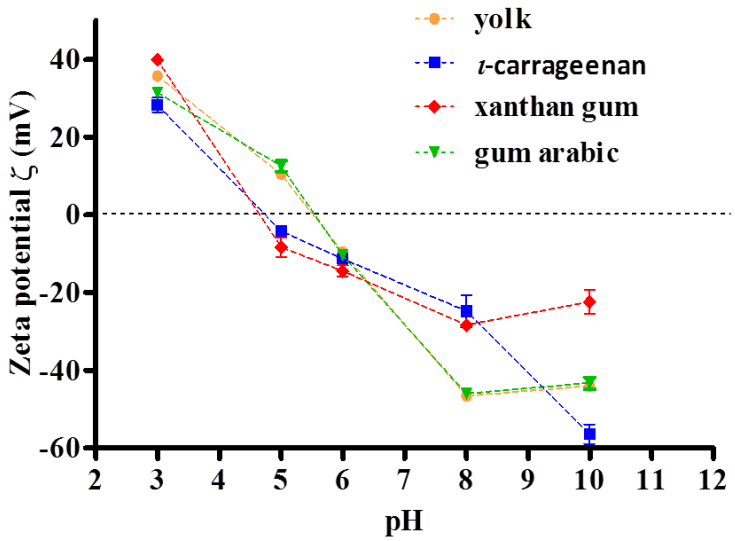

Figure 7.3. Average zeta potential (ζ) of suspensions prepared from egg yolk: (-●-) egg yolk (-■-) ι-carrageenan/yolk (-♦-) xanthan gum/yolk (-▼-) gum arabic/yolk as a function of pH (n=3), at fixed concentration of 0.4% of polysaccharides.

It was reported that sulphated polysaccharides such as carrageenans, but not carboxylated polysaccharides such as xanthan gum and pectin, may have some interactions with proteins above their isoelectric points (Samant et al., 1993). Sulphated polysaccharides such as carrageenans interact more strongly with proteins than carboxylated polysaccharides such as xanthan gum or pectin, because the electrostatic interactions between ($–NH_3^+$) of proteins with ($-OSO_3^-$) of carrageenans is stronger than that with ($-COO^-$) of polysaccharides such as xanthan gum and pectin (Doublier et al., 2000).

7.3.2.2 Particle size

Although the zeta potential was almost equal at pH 6 and 5 between xanthan gum/yolk and *ι*-carrageenan/yolk suspensions, the average particle size of *ι*-carrageenan/yolk suspensions was considerably higher than those of the other two polysaccharides at pH lower than 6 (Fig. 7.4). In addition to electrostatic interactions explained above, different rheological properties of three polysaccharides might change at different pH conditions, which also contribute to the observation. The particle size decreased dramatically with increasing pH above 6 and there was no difference at pH 8 and 10. Suspensions of *ι*-carrageenan/yolk showed an average particle size of about 540 μm at pH 3, which decreased with increasing pH and reached its minimum of ~35 μm at pH 8. Particle size of xanthan gum/yolk suspensions increased from 38 μm at pH 3 to 115 μm at pH 5 but decreased at increasing pH. The effect of pH on the particle size of gum arabic/yolk suspensions was negligible. For xanthan gum/yolk suspensions, the largest particle size was at pH 5 and then 6, the values close to

egg yolk and LDL isoelectric points. The overall decrease of particle size at alkaline pH is probably due to disassociation of the HDL and phosvitin complex, leading to solubilisation of granules as well as possible changes in polysaccharides structure (Causeret et al., 1991).

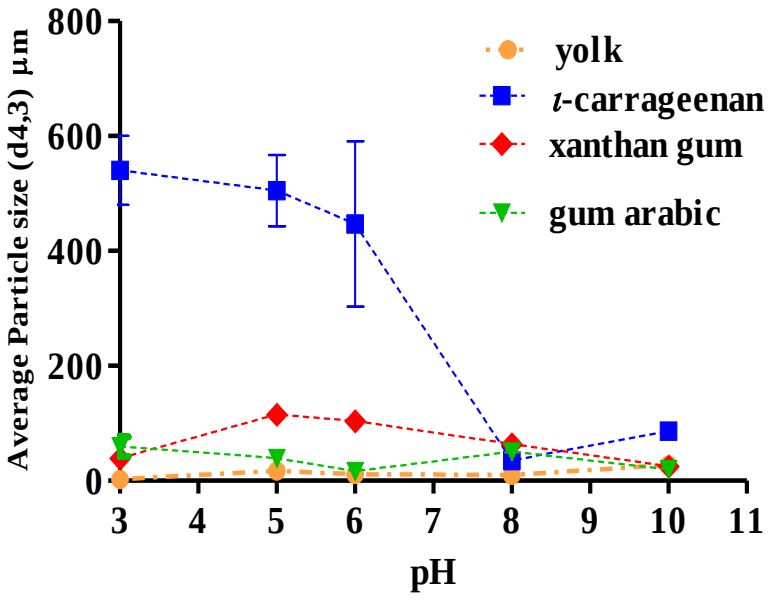

Figure 7.4. The average particle size of egg yolk suspension in the presence of 0.4% concentration of gum arabic, *ι*-carrageenan and xanthan gum at different pH levels (n=3).

7.3.2.3. Phase separation behaviour

Two-fold diluted egg yolk as expected formed two distinct phases; an insoluble pellet called granules and a liquid phase referred to as plasma. After centrifugation of suspensions of different yolk/polysaccharide treatments at different pH levels, only xanthan gum/yolk suspensions formed three distinct phases at pH 6. These phases were cream on top, aqueous phase in the middle and pellet at the bottom (Fig.7.5), the remaining yolk/polysaccharide treatments

formed either biphasic or monophasic systems. Shah and Singh (1992) also obtained a cream fraction rich in lipids after mixing carboxymethyl cellulose (CMC) with plasma fraction obtained from egg yolk 2-fold dilution. However, mixing CMC directly with yolk suspension at its natural pH, co-precipitated LDL particles along with other proteins into pellet fraction and no semi-solid cream was formed. In this study, upon phase separation, cream phase comprised considerable amount of lipids.

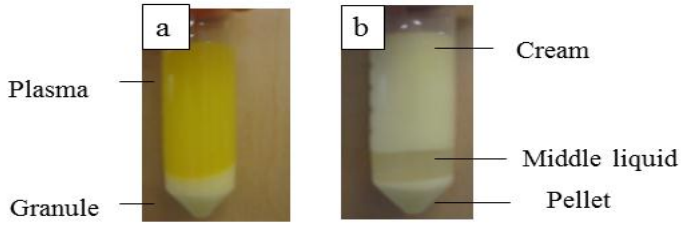

Figure 7.5. Phase separation for egg yolk dilution at 0.4% polysaccharide concentration and pH 6, a) biphasic separation for gum arabic/yolk or *ι*-carrageenan/yolk, b) triphasic separation for xanthan gum/yolk suspension.

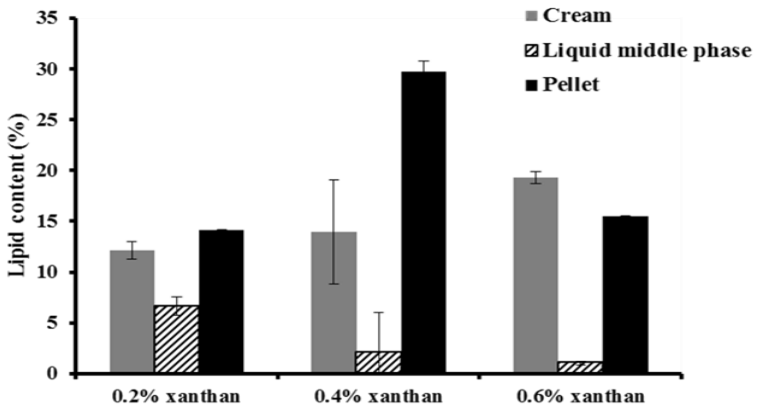

Figure 7.6. Lipid contents in the cream, middle liquid phase and pellet obtained from xanthan gum/yolk suspensions at 0.2%, 0.4% and 0.6% xanthan gum concentration (fresh weight basis (n=2).

7.3.3. Characterisation of cream phase of xanthan gum/yolk suspension

7.3.3.1. Protein profile of fractions from yolk-xanthan gum interactions at pH 6

Protein profile of the cream phase formed by the addition of xanthan gum was examined by SDS-PAGE and compared to those of the "pure' LDL isolated from yolk, as well as the plasma fraction of egg yolk (Fig.7.7). As expected, SDS-PAGE results showed a high level of similarity between the protein profiles of the cream from xanthan gum, the plasma and LDL. This similarity may arise from the fact that over 70% of plasma dry matter is composed of LDL. LDL apoproteins were reported to have a molecular weight distribution ranging from 240, to 223, 203, 130, 93, 78, 68, 65, 62, 60, 32, 31, 23, 22, 17, 16 and 11 kDa (Aluko et al., 1998; Guilmineau et al., 2005).

A major band over 250 kDa, being an apoprotein from LDL, was observed in all three samples. Livetins are soluble proteins present in the plasma fraction along with LDL. Livetins in plasma are comprised of α-, β- and γ-livetins. γ-Livetin subunits have been reported to have molecular weights of 130, 104, 78 and 29 kDa based on SDS-PAGE analyses, while α- and β-livetins have molecular weights of 78, 40 and 38 kDa (Guilmineau et al., 2005). According to Moussa et al. (2002), LDL extracted based on their method, without further purification by gel filtration, has a purity of 97%, meaning there may be some impurities from livetins. However, despite the high level of similarity between the cream and the "pure" LDL, three more bands with molecular weights of 110, 78 and 29 kDa

were observed for the cream phase obtained from xanthan gum/yolk suspension (Fig. 7.7, Lane C1).

It seems that in addition to LDL, some livetins, most probably γ-livetin, were also present in the cream phase. Cream and subnatant liquid fractions obtained from plasma/xanthan gum were carefully separated for further protein profile analysis as shown in Figure 7.7, lanes C2 (cream) and S (liquid subnatant). Protein profiles showed that 10 major bands were present in the subnatant, which could not be absorbed into the cream. These bands had molecular weights of 78, 65, 43, 38, 33, 26, 24, 21, 13 and 10 kDa. Bands with molecular weights of 78, 40 and 38 kDa could be from α-and β- livetins, while the bands of 21 kDa as well as 78 kDa were from γ-livetin (Guilmineau et al., 2005; Jolivet et al., 2008). Protein bands with molecular weights of 104 and 24 kDa may be related to γ-livetin, which remained in the cream fraction (Aluko et al., 1998). Two bands with molecular weights of 26 and13 kDa present in the subnatant fraction might come from LDL. Interestingly, three bands of 110, 78 and 29 kDa, observed in the cream fraction from xanthan gum/yolk, were absent in the cream formed by xanthan gum/plasma treatment, meaning the purity of LDL obtained from the plasma fraction was higher than that of whole egg yolk. Overall, the results of the SDS-PAGE analysis indicated that LDL was separated into the cream phase; however, some livetins such as γ-livetin may also be present in the cream along with LDL.

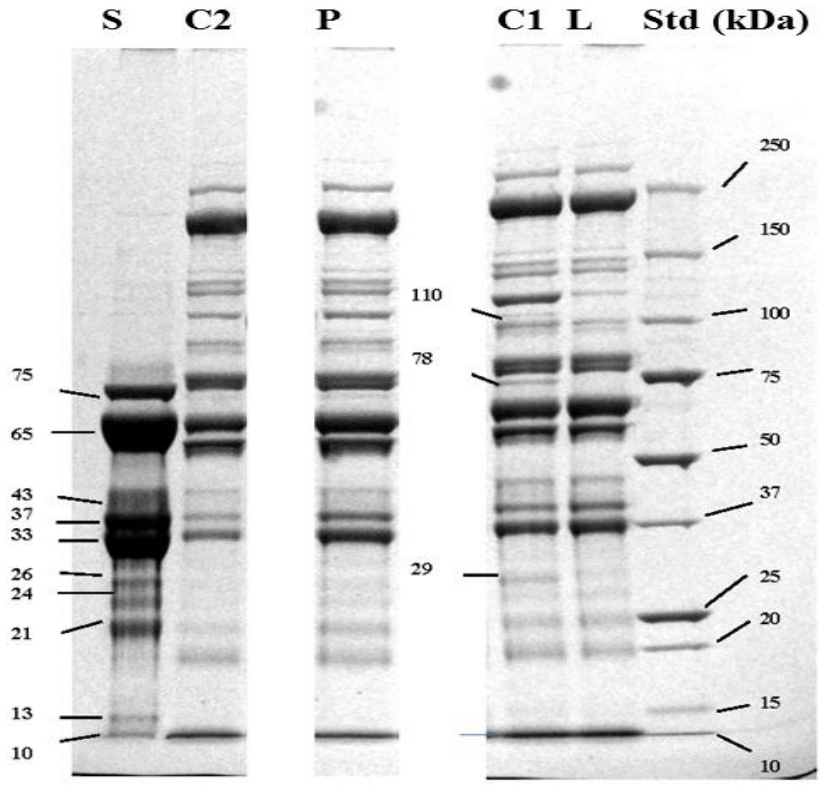

Figure 7.7. Protein profile of different fractions of egg yolk Protein profiles of different fractions of egg yolk : (L) LDL, (C1): cream (yolk/xanthan gum) (P): plasma, (C2) cream from plasma/xanthan gum, (S): subnatant-liquid from plasma/xanthan gum.

7.3.3.2. Microscopic analyses of cream derived from yolk-xanthan gum

Lipid determination and the protein profile analysis confirmed that LDL was separated in the cream fraction (Figs. 7.6 and 7.7). Further microscopy analysis was performed to localize and confirm the co-presence of xanthan and LDL in the cream as well as to obtain additional information about the structural arrangement between xanthan gum and LDL and other major egg yolk components present in the cream.

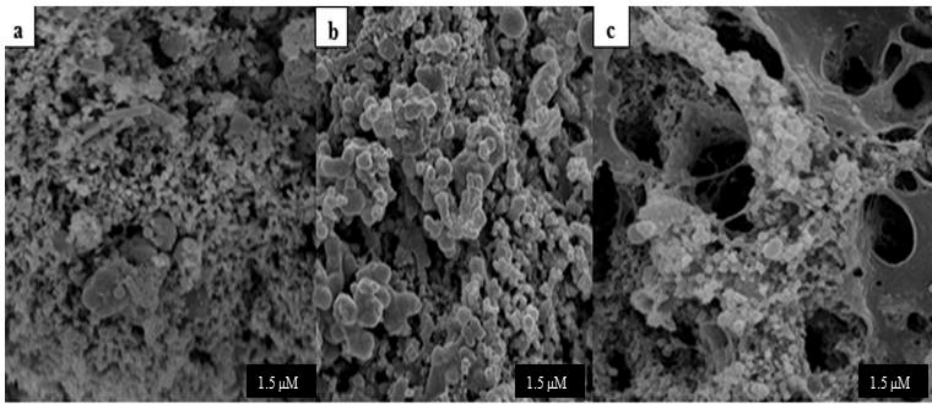

Figure 7.8. SEM micrographs for different fractions of egg yolk: a) intact fresh egg yolk , b) "pure" LDL, c) cream fraction from xanthan gum/yolk. Bar=1.5 µm

Scanning electron micrographs of egg yolk, LDL and cream are shown in Figure 7.8. Egg yolk seems to have a more heterogeneous structure (Fig. 7.8a), while the "pure" LDL seemed to be more compact and homogeneous with no prominent network structure (Fig. 7.8b). However, the cream phase (Fig. 7.8c) obtained after xanthan gum addition clearly showed strands of a network-like structure, attaching to aggregated particles, while such a network was not present in LDL or egg yolk. SEM results demonstrated that egg yolk components were trapped in the network formed by xanthan.

The presence of proteins and polysaccharide and their arrangement in the cream were examined by CLSM (Fig. 7.9a,b). With the use of dyes, xanthan gum was visualised in green while proteins and lipids were in red and bright yellow-orange, respectively. CLSM micrographs indicated that xanthan gum formed a network-like structure where egg yolk components were trapped inside the xanthan gum network. A higher magnification image (Fig. 7.9a) showed that the egg yolk components adhered to xanthan gum network seemed to be aggregated

together. Bright yellow and orange spots were dominant, indicating the presence and accumulation of high amounts of lipid-rich components, i.e. LDL particles in the cream phase.

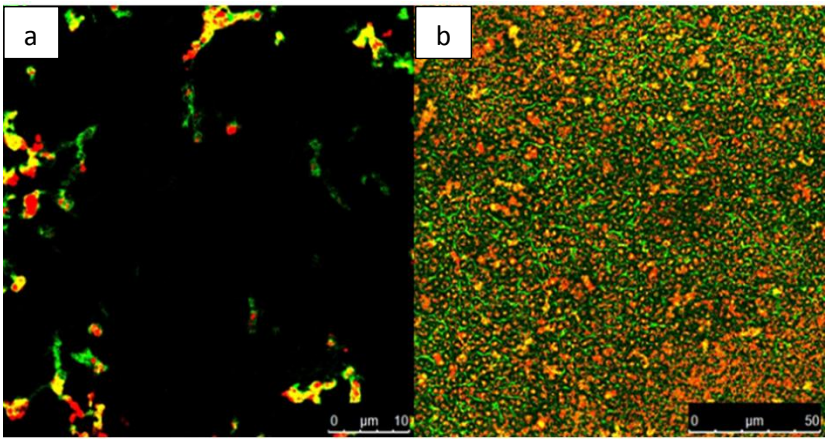

Figure 7.9. Confocal micrographs of cream fractions obtained from egg yolk/xanthan gum suspension at pH 6. Bar lengths (a) 10 µm and (b) 50 µm.

7.4. Proposed interactions between egg yolk and xanthan gum

Possible phase separation after mixing two biopolymers is the outcome of the several factors such as polysaccharide and protein types, pH, ionic strength and dilution factor (McClements, 2006). When two biopolymers are mixed, they may form monophasic or biphasic systems. Monophasic systems can be formed for two reasons: a) either two biopolymers do not have any attractive interactions and do not form complexes; but both are co-soluble in the solution, or b) the interactions between biopolymers result in the formation of new complexes, which are still soluble in the solution. Phase separation and biphasic system formation in a mixture of biopolymers can happen due to associative or segregative interactions. If two biopolymers are not thermodynamically compatible, after mixing they form two different phases in the solution. In this

case, entropic contribution due to incompatibility between two polymers is more than enthalpic ones. This may happen if two biopolymers contain the same charge and repulse each other. Incompatibility may promote self-association among biopolymers of the same type and cause depletion flocculation and phase separation (Polyakov et al., 1997). In the second case, if two biopolymers contain opposite charges, then attractive interactions result in the formation of complexes, which are not soluble, then phase separation will occur.

In addition to structural changes that happen in egg yolk with pH manipulation, very acidic or alkali conditions may also change polysaccharide structure and its functional groups charge and availability. For example, xanthan gum at acidic pH is less viscous and its carboxyl groups can convert from ionized groups to unionized ones (Phillips and Williams, 2000). Neutralization of the xanthan gum can re-ionize the polysaccharide. Therefore, changes in yolk structure as well as polysaccharides influence their physicochemical properties and interaction types.

As shown in Figure 7.3, the net charge of egg yolk suspensions after xanthan gum or carrageenan addition became slightly negative at pH 6, which may interact with the slightly positive charged LDL at pH 6. However, other interactions, such as hydrophobic and hydrogen bonding can also be responsible for proteins and polysaccharides interactions. Previously, Goodman and Shafrir (1959) reported that the presence of weak hydrophobic groups in human plasma lipoproteins could promote hydrophobic interactions with polysaccharides. In egg yolk, over 40% of amino acids of LDL apoproteins are hydrophobic and some

lipids on LDL surface may also have hydrophobic properties (Tsutsui and Obara, 1982; Anton et al., 2003), which may interact with hydrophobic cavities formed by the hydrophobic pryuvate chains of xanthan gum on its ordered double helix conformation (Jouquand et al., 2008). Since PL are present on the surface of LDL particles, there is always the possibility of interactions between these amphipathic compounds and hydrophilic sites of xanthan gum, which may form insoluble complexes (Kozarac et al., 2000).

Electrostatic interactions between negatively charged xanthan gum and positively charged patches of LDL at pH 6 can be the initial force promoting the interaction between xanthan gum and LDL. As LDL itself contains a high percentage of hydrophobic apoproteins, self-aggregation among LDL particles is also possible. In fact, CLSM of cream showed some lipid-rich aggregates, attaching to the xanthan gum strands. It has been reported that while electrostatic interactions can be the initial factor for interaction between polysaccharides and biopolymers, hydrogen or hydrophobic interactions may play a greater role in the next steps as the related functional groups get more chance for anchoring (Doublier et al., 2000).

Xanthan gum solutions have considerably higher viscosity at similar concentrations compared to the other polysaccharides tested in the study. Below transitional temperature, its helix-like structure can be considered as a rigid rod, which also forms intermolecular bonds. The intermolecular associations form a complex network of weakly bound molecules, which makes xanthan solutions to be very viscous at rest. Xanthan gum solutions also exhibit shear-thinning

behaviour, indicating that viscosity decreases with shear rate (Phillips and Williams, 2000). Upon mixing and centrifugation, xanthan gum viscosity decreases, facilitating the possible interactions between yolk and xanthan gum. After the removal of external force, xanthan gum solution becomes viscous again and re-forms a gel-like network. LDL trapped in the xanthan gum network will form a cream on top due to the density difference between cream and the other fractions. As major components of LDL are lipids, LDL are lighter than water. Therefore, LDL entrapped in the xanthan gum network is separated due to the density differences between cream and the rest of the suspension. A schematic summary of the suggested mechanism for LDL separation as described above has been shown in Figure 7.10.

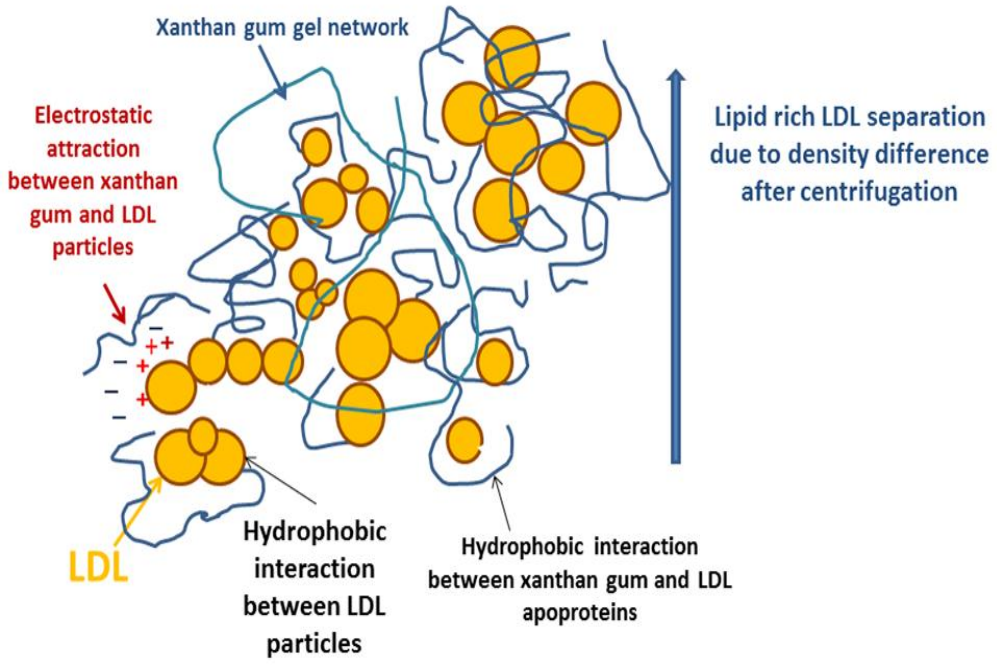

Figure 7.10. Suggested mechanism for LDL separation from egg yolk after xanthan gum addition to egg yolk.

7.5. Conclusions

The nature of polysaccharide and pH condition play great roles in dictating the types of interactions between egg yolk and polysaccharides and therefore phase separation behaviour. In this study, among the three polysaccharides investigated, i.e. gum arabic, *ι*-carrageenan and xanthan gum, only xanthan gum showed distinct phase separation behaviour. A semi-solid cream rich in LDL was successfully separated from 2-fold diluted egg yolk using xanthan gum at egg yolk natural pH 6 and close to LDL isoelectric point. At this condition, granules are insoluble and have the least chance for interactions with xanthan gum. A combination of electrostatic and hydrophobic forces as well as the rheological properties of xanthan gum were suggested as possible reasons for the unique separation of LDL with xanthan gum, but not with other two polysaccharides. However, further research is required to understand the exact nature of interactions between LDL and xanthan gum and subsequently to develop possible approaches to separate intact LDL from the polysaccharides, through for example ionic strength or pH manipulation or other approaches, for further potential applications.

7.6. References

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8. Conclusions and recommendations

Egg yolk fractionation through 10-fold water dilution can be an approach for IgY separation from egg yolk, while a major part of the lipid compounds remaining in the pellet (Kwan et al., 1991). The leftover pellet contains 90% of lipids and PL of egg yolk. The pellet can be applied as an emulsifier in various food products; however, there is a lack of detailed information about the physicochemical properties and possible changes in the functional properties of the pellet after IgY extraction through 10-fold dilution compared to those of the intact yolk. Also, the pellet can be further processed to extract high-value PC for nutraceutical and pharmaceutical applications. However, PL extraction and fractionation from egg yolk at industrial scale is not well defined and is quite a complicated process, with the use of hazardous organic solvents for de-oiling and purification of PL (Schneider, 1989; Gadkowski et al., 2012). Supercritical carbon dioxide (SC-CO_2) as a "green" solvent has been applied successfully for the extraction of several high-value lipids at industrial level (Temelli, 2009). However, due to the low recovery of PL of egg yolk using SC-CO_2 technology this technique has not been considered for scale-up purposes (Shah et al, 2004; Aro et al, 2009). Considering the structure of egg yolk and the position of PL in this structure (Burley and Vadehra, 1989) enzymatic hydrolysis can be an approach to increase the recovery of PL from egg yolk using SC-CO_2+ethanol.

Meanwhile, since low density lipoproteins (LDL) comprise over 70% of egg yolk dry matter and over 90% of lipids, including PL are in LDL (Anton, 2007), separation of these particles through non-solvent based techniques can be a

new approach that may result in the introduction of new extraction techniques as well as new applications for these products. Biopolymer interactions, for example between polysaccharides and proteins, can be a promising approach to extract high-value compounds in organic solvent free environment. Therefore, the overall objectives of this thesis research were to characterize egg yolk emulsion properties after IgY extraction, impact of enzymatic digestions on the recovery and purity of PL through SC-CO_2 technology and to separate LDL particles as nanoparticles rich in PL.

In the first study, physicochemical aspects of egg yolk after IgY extraction through 10-fold water dilution was studied. This study showed that 90% of egg yolk lipids were recovered in the pellet after 10-fold dilution and the separation of soluble fraction (Chapter3). The leftover pellet had physicochemical properties distinct from intact egg yolk in terms of emulsion and rheological properties. Emulsions prepared from the pellet were less stable since the coalescence index was significantly higher than that of intact yolk. Strain sweep test for the pellet and yolk showed that the pellet had higher G' and G" values, an indication of the stronger structure of the pellet. While egg yolk exhibited a Newtonian behaviour (n=1.02), the pellet showed shear thinning behaviour (n=0.33). The results of emulsion and rheological studies provide useful information regarding the technological differences and the processing aspects, which should not be overlooked when the pellet is used as a substitute for intact egg yolk as an emulsifier.

Since over 90% of lipid and PL of egg yolk remain in the pellet, it can be further processed to recover high-value PC for nutraceutical and pharmaceutical applications. The low recovery of PC extracted with SC-CO_2+ ethanol from dry yolk in previous studies has been a major barrier for scale up purposes. Therefore, a combination of enzymatic hydrolysis and a two-step SC-CO_2 extraction technique was investigated as an approach for de-oiling and extraction of PL without using hazardous organic solvents.

First, the use of six proteases, all combined with Lipase AY30, on emulsion properties, phase separation behaviour and PL recovery in cream phases obtained after enzymatic treatment were investigated (Chapter 4). Results showed that emulsions prepared from the pellet hydrolysed with Protease P, Protease II and Protease M had larger oil droplet size than pellet or hydrolysates from Protease A, Protex 7L and Protex 51FP. Particle size distribution of hydrolysates also indicated that the average particle size for hydrolysates of Protease II and Protease P were the highest among all hydrolysates, the pellet or the intact yolk.

PL analyses showed that a portion of PL was probably hydrolysed during enzymatic treatment of the pellet. While no LPC could be detected in the intact yolk or pellet fractions, enzymatic hydrolysis of pellet with Protease P and Protease M together with Lipase AY30 increased the LPC content to detectable levels. The highest recovery of PC and PL was obtained with Protease P where 98% of PL and 81% of PC from fresh yolk were recovered in the cream fraction in this study. Therefore, among the studied enzymes, Protease P + Lipase AY30 could be the most suitable enzyme for PL extraction using SC-CO_2 due to its

weak emulsion properties and the highest PL recovery in the cream. The second option was the cream obtained from the Protease II + Lipase AY30 treatment, where in addition to weak emulsion properties of the hydrolysate, 77% of PL and 75% of PC from the yolk were recovered in the cream fraction.

The recovery of PC and PL of dry yolk using SC-CO_2+ethanol extraction was 46.8% and 48.8%, respectively (Chapter 5). The recovery (yolk-based) of PC (85%) and PL (86.5%) in the extract significantly increased for dry cream of Protease P + Lipase AY30. On the other hand, the recovery of PC from dry cream as the feed to the extractor was 94%. Despite the considerable increase in PC recovery, enzymatic treatment did not significantly improve the purity of PC and PL.

Further studies at two moisture levels (20% and 45%) of cream showed that overall enzymatic hydrolysis could improve the extractability of PC and PL, even in the presence of high levels of moisture (Chapter 6). The recovery of PC from cream to extract was 90% and 106% for creams with 45% and 20% moisture content, respectively. The recovery of PC for the whole process (yolk based) was 69.8% and 61% for the cream with 20% and 45% moisture content, respectively. The highest purity of PC and PL (84% and 103%, respectively) was for the cream with 20% moisture content. It seems that the combination of ethanol and certain levels of water acted as an entrainer for selective extraction of PC and PE from the cream with intermediate moisture content.

Weak structure of cream and higher solubility of PC in SC-CO_2+ethanol increased the recovery of PC. Enzymatic digestion could increase PE recovery

from 2% in dry yolk to about 40% in dry cream and cream with 20% moisture; however, lower solubility of PE in SC-CO_2+ethanol was the reason for lower recovery of PE.

This thesis research demonstrated that enzymatic treatment followed by SC-CO_2+ethanol extraction gave promising results in terms of both recovery and purity. However, not all proteolytic enzymes can be suitable for this purpose. A combination of Lipase AY30 and specific proteases could reduce drastically the PL amount in final hydrolysate, which based on our knowledge has not been reported before and may initiate further studies in the future. Identification and quantification of impurities such as lipids and possible peptides extracted at different treatments also could be an interesting aspect for further studies.

The separation of LDL particles from yolk as a distinct phase through polysaccharide-egg yolk interactions was also investigated (Chapter 7). LDL as the major components of egg yolk contains over 90% of egg yolk lipids and PL. This study showed that based on polysaccharide type and applied pH, egg yolk LDL could be separated as a distinct cream phase from 2-fold diluted yolk. Among the three applied polysaccharides (gum arabic, carrageenan and xanthan gum), xanthan gum was the polysaccharide that resulted in LDL separation as a distinct cream fraction at pH 6. The co-presence of xanthan gum and LDL particles was confirmed via different microscopic techniques (SEM and CLSM). SDS-PAGE profile of the cream fraction indicated that some livetins (most probably IgY) was present along with apoproteins of LDL. However, more accurate techniques such as mass spectroscopy may be required to identify the

protein types absorbed in the xanthan gum network along with LDL. This study suggests that a combination of electrostatic and hydrophobic interactions may induce primary attraction between LDL and xanthan gum. Further studies may focus on more thorough understanding of the nature of interactions between xanthan gum and absorbed LDL in the cream fraction as well as possibilities to separate LDL from xanthan gum with the least damage to the LDL structure. Overall, this study showed that the polysaccharide/yolk interaction is a promising approach for isolation of LDL from egg yolk. Better understanding the behaviour of xanthan gum under different ionic strength or pH conditions, which may reduce the viscosity of xanthan gum, may help to separate LDL from the polysaccharide.

Currently, majority of PL used for various industrial applications is obtained from soy lecithin, which is a by-product of soybean oil refining. However, the nutritional value of egg yolk PL is higher, considering the fact that choline content in soybean PL is almost three times lower than that of egg yolk PL. PL extraction from egg yolk using conventional techniques or previously reported SC-CO_2 extraction has limitations. This thesis research focused on novel extraction techniques based on enzymatic hydrolysis technology to improve the extractability of PL from egg yolk. To the best of our knowledge, there is no previous study that has evaluated the impact of enzymes to increase the recovery of PL from egg yolk. The enzymes, Protease P and Lipase AY30, employed in this study are food-grade enzymes with extensive applications in the food industry to develop aroma in dairy and meat products. Although PL extraction using SC-CO_2 still requires ethanol as a co-solvent for PL extraction, there is no restriction

for ethanol usage in food processing due to its GRAS status. The molar fraction of ethanol (8%) used as co-solvent in this study was lower than that applied in previous studies (12%) (Shah et al., 2004; Aro et al., 2009). Results from this research indicate that SC-CO_2 extraction of PL from hydrolysed cream with intermediate moisture showed quite good recovery and high purity of PL. However, possible co-extraction of water and quantification of its amount in the final extracts are important aspects, which were not covered in this study and recommended for future work.

This thesis research also showed that xanthan gum has potential for LDL separation from yolk. Considering the fact that no pH adjustment or further egg yolk fractionation to plasma and granules were necessary to isolate LDL from egg yolk, this technique can be a very simple and fast approach for LDL separation from yolk matrix. Whether separated LDL can be feasible as a feed material for PC extraction using SC-CO_2 technology is also recommended for future works.

Overall, this thesis research and its findings are a great contribution to the egg producers, processors and related industries through introducing novel approaches to exploit high-value bioactive compounds such as PL and IgY, optimizing the recovery of PL and opening new perspectives for potential new applications. This research meets current demands for more sustainable and eco-friendly techniques for PL extraction through eliminating the use of hazardous organic solvents and providing safe and “natural” ingredients for products such as infant formula. The peptides left as a by-product after lipids extraction by SC-CO_2

are free from hazardous organic solvents, are a good protein source and may have some distinct bioactive properties.

8.1. References

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