

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

Investigations on the emulsifying properties of egg white protein

**A thesis presented in partial fulfilment of the requirements for
the degree of Master of Food Technology
at
Massey University, Auckland, New Zealand**



MASSEY UNIVERSITY

**Amarachi Delight Onyemachi
2018**

ABSTRACT

Egg white proteins (EWP) have excellent foaming and gelling functional properties. However, their emulsifying properties are considered poor when compared to soy proteins or milk proteins. Some studies have attributed the poor emulsifying properties to the hydrophobic amino acid groups buried deeply in the interior of the protein conformational structure which is crucial for emulsification. Several methods, such as heat treatment, acid/acid-heat treatment, Maillard reaction, phosphorylation and enzymatic hydrolysis, have been used by some researchers to improve the emulsifying properties of EWP. Preliminary experiments carried out in this study showed that oil-in-water (O/W) emulsions prepared with egg white liquid (EWL) generated lots of visible large aggregates, which no other study has reported. Therefore, it was important to investigate the factors responsible for the formation of these aggregates. Investigations into improving EWP's emulsifying properties could offer opportunities in developing unique and well-defined egg white-based emulsions.

The objective of this research project was to produce egg white emulsions with little or no aggregates. This thesis comprises three main parts. The first part focused on the effects of pH and heat treatment on protein aggregation and partial denaturation of proteins in EWL. The second part investigated the effects of heat treatment, oil concentration and protein concentration on the reduction of large visible aggregates in emulsions prepared with EWL. The third part studied the effect of enzymatic hydrolysis on the degree of hydrolysis and emulsifying properties of EWP hydrolysates. The emulsifying properties of original EWP and EWP hydrolysates were characterised in terms of size and zeta (ζ)-potential of emulsion droplets and emulsion stability (e.g. turbidity, microscopic examination and phase separation).

Firstly, an experimental study was carried out to evaluate the effect of pH on protein aggregation and precipitation in EWL containing different protein concentrations (0.5, 1, 2, 3, 4, 5 and 10% w/w). It was found that at all the protein concentrations used and at pH less than around 5, ζ -potential values were all positive but decreased as pH increased from 2 to 5. At pH 5, ζ -potential was close to zero (this is the pI of most egg white proteins), while, at pH levels above 5, ζ -potential became negative and increased as pH increased from pH 5 to 11. The spectral absorbance (turbidity) of emulsion samples was also

measured at 600 nm which revealed that for all protein concentrations, turbidity was observed to be higher at acidic pH of 3, 4 and 5, indicating the aggregation of EWP. At alkaline conditions of pH 7, 8, 9 and 10 the EWL solutions remained to be transparent. The effect of heat treatment and holding time on the denaturation of EWP in EWL was also studied at different temperatures (57-62°C) and heating times (0-19 minutes). Higher turbidity due to protein aggregation was observed as temperature increased from 57 to 62°C and the heating time increased from 5 to 19 minutes. It is therefore concluded that EWL can be safely pasteurized with little or no denaturation or aggregation at around 57-58°C for less than 5 minutes. At 60°C, it was observed that EWL began to thicken and after 5 minutes coagulation and gelation occurred rapidly.

Studies were also carried out to determine the cause of visible large aggregates formed in emulsions prepared with EWL using various factors, such as heat treatment, oil concentration and protein concentration. It was found that heat treatment (60°C for 30 minutes) of 1% (w/w) EWP solution prior to homogenisation had no effect on reduction of aggregates in emulsions containing 5, 10, 15 and 20% (w/w). However, the formation of aggregates was reduced significantly as oil concentration was reduced to 5%. Therefore, the effect of lower oil concentrations (1, 3, 5, 6, 7 and 10% w/w) on the formation of aggregates in emulsions prepared with 1% or 3% EWP concentrations was also investigated. Little or no visible aggregates were formed when emulsions were prepared with 1% EWP and $\leq 5\%$ oil or 3% EWP and 1% oil. Therefore, the results indicated that both protein and oil concentrations played a significant role in the formation of visible aggregates in emulsions prepared with EWP as an emulsifier.

The effect of EWP concentrations (0.1, 0.3, 0.5, 0.8, 1 and 2% w/w) on the formation and properties of 5% oil emulsions at ~pH 8 was then investigated. It was discovered that little or no aggregates were produced in emulsions when prepared at 0.1-1% EWP while large aggregates were formed at 2% EWP concentration. The size of emulsion droplets was observed to increase significantly from 242.1 to 703.7 nm as protein concentration increased from 0.1 to 2%. ζ -potential was however not significantly affected by protein concentration and ranged from -35.3 to -39.2 mV. The emulsions prepared were also heat treated at 60-90°C for 30 minutes. No sign of instability with a significant change in the size of emulsions due to heat treatment was observed from all emulsion samples prepared at different EWP concentrations (0.1 - 2%). However, phase separation of the emulsions

was observed upon freezing at -20°C and thawing at 4 and 20°C, respectively, at all protein concentrations used. Also, the stability of emulsions was affected by the addition of salts, such as CaCl₂ (5-100 mM) and NaCl (50-600 mM), with an increase in droplet size and phase separation. However, the emulsions were relatively more stable to salt-induced flocculation, especially against NaCl, at higher protein concentration (1-2%) than lower protein concentrations (0.1-0.8%). Lastly, the effect of pH 2-10 was also determined from the emulsions prepared at 1% EWP and 5% oil. Extensive droplet aggregation was observed at pH 4 and 5 as expected which is around the pI of most egg white proteins. On the other hand, it was not observed at extremely acidic pH 2.0 and alkaline pH 9-10 and in the control emulsion prepared at pH 8.3.

In another part of the study, the effects of enzyme type (bromelain, ficin and papain), enzyme concentration (0.3, 0.5, 1, 2 and 4% w/w; enzyme/substrate (E/S) ratio) and hydrolysis time (0, 30, 60 and 120 minutes) on the degree of hydrolysis (DH) of EWP were investigated by diluting EWL containing 10% EWP to different EWP concentrations followed by adding enzymes into the EWL solutions. DH was observed to increase significantly ($p < 0.05$) with increasing enzyme concentration and hydrolysis time. A significant difference ($p < 0.05$) among the different types of enzymes was only observed from the samples with 4% E/S ratio at 120 minutes of hydrolysis time. Papain yielded the highest DH of 7.69% while bromelain and ficin yielded similar DH levels of 5.03% and 4.99%, respectively. The results of SDS-PAGE revealed that the protein bands corresponding to ovalbumin and ovotransferrin disappeared due to their enzymatic hydrolysis into smaller peptides but it was not significantly different between the samples treated with different E/S ratios and hydrolysis reaction times.

The effects of enzyme concentration, DH and hydrolysis time on the emulsifying properties of hydrolysed EWP prepared with bromelain and ficin were investigated. Surprisingly, enzymatic hydrolysis significantly improved the appearance of emulsions prepared with EWL containing hydrolysed EWP by producing an emulsion free of aggregates compared to the control emulsions prepared from original EWP which had lots of large aggregates in it. For example, emulsions containing 10% oil and various EWP concentrations (1, 5 and 10%) prepared with hydrolysed EWP (4% E/S, DH 5.16%) yielded smaller droplet size (0.66-0.98 μm) than those of original EWP emulsions (1.22-39.35 μm). However, phase separation occurred immediately after preparation at all protein concentrations (1, 5 and

10%) used while phase separation occurred in only emulsions stabilised with 5 and 10% original EWP. When the emulsions were heat treated at 60-90°C for 0-30 minutes, gelation occurred in the emulsions prepared with 5 and 10% EWP concentrations while the emulsions prepared with 1% EWP had no gelation but had aggregation and phase separation after heat treatment. Emulsions prepared with 1% E/S ficin (DH 4.03% and 4.96%, respectively, after 2 and 4 hours of hydrolysis time) yielded smaller droplets size (0.75-0.87 μm) than droplet size (6.40-7.37 μm) of emulsions prepared with 1% E/S bromelain (DH 4.10% and 4.87% after 2 and 4 hours of hydrolysis time). Droplet size decreased as hydrolysis time increased from 2 to 4 hours for both ficin and bromelain hydrolysates with phase separation occurring the following day after the preparation of emulsions. Thus, DH and enzyme type had some influence on the emulsifying properties of EWP hydrolysates.

In conclusion, this study demonstrated that egg white emulsions can be prepared with little or no aggregates using low oil ($\leq 5\%$) and low protein (1%) concentrations and by enzymatic hydrolysis of EWP. Emulsions containing 5% oil prepared with a relatively higher protein concentration (1-2%) were more stable to destabilization to ionic strength (salt concentration), especially against NaCl. These could lead to production of egg white protein based-emulsions with distinct appearance and characteristics.

ACKNOWLEDGEMENTS

First, my appreciation goes to the Almighty God, the author and finisher of my faith; the giver of wisdom and strength. I am nothing without you.

My utmost appreciation goes to my supervisor Dr Sung Je Lee for his thorough suggestions, intellectual expertise, constructive feedback and professional guidance from the beginning to the completion of my research project. I have learnt a lot working under his supervision.

I am grateful to Dr Jasper Mbachu and Dr Tony Mutukumira for their constant encouragement and advice.

I would like to express my gratitude to the Lab managers and technicians of the Massey Institute of Food Science and Technology (MIFST); Dr Kenneth Teh, P. C. Tong and Jia Shi for training me in laboratory protocols and use of equipment.

My appreciation goes to the New Zealand Ministry of Foreign Affairs and Trade for the opportunity given to me to study at Massey University through the New Zealand Aid Development Scholarship. Heartily, I would like to express my sincere gratitude to Sylvia Hooker, the Team Leader and Jamie Hooper, Anita Albert and Dave Broderick, the Support Officers for their immeasurable support during my entire stay in New Zealand. I also wish to acknowledge the MIFST's Postgraduate Research Support Committee for supporting my research project.

My gratitude also goes to my parents, siblings and in-laws, for their prayers, encouragement and support which kept me strong throughout my programme.

Finally, my appreciation goes to my lovely husband, Dr Chidozie Anyiro for his support, encouragement, prayers, and inspiration.

TABLE OF CONTENTS

ABSTRACT	i
ACKNOWLEDGEMENTS	v
TABLE OF CONTENTS	vi
LIST OF TABLES	xi
LIST OF FIGURES	xiii
LIST OF APPENDICES	xvii
LIST OF SYMBOLS	xviii
LIST OF ABBREVIATIONS	xix
Chapter 1. Introduction	1
Chapter 2. Literature Review	4
2.0 Egg white protein	4
2.1 Nutritional composition of egg white.....	4
2.2 Structure and composition of egg white.....	5
2.3 Physicochemical properties of egg white.....	6
2.4 Major egg white proteins and their characteristics.....	8
2.4.1 Ovalbumin	8
2.4.2 Ovotransferrin (Conalbumin)	9
2.4.3 Ovomucin	10
2.4.4 Lysozyme.....	11
2.4.5 Ovomuroid	13
2.4.6 Ovoflavoprotein.....	13
2.4.7 Ovoglobulin	13
2.4.8 Ovoinhibitor.....	13
2.4.9 Avidin	13
2.4.10 Cystatin.....	14
2.5 Functional properties of egg white protein	14
2.6 Food emulsions	15
2.6.1 Food emulsion properties	15
2.6.1.1 Droplet size and size distribution.....	16
2.6.1.2 Droplet electrical charge	16
2.6.1.3 Droplet microstructure	17
2.6.1.4 Emulsion appearance	18

2.6.1.5 Rheological properties	19
2.6.2 Factors influencing protein-stabilized emulsions	20
2.6.2.1 Thermal processing.....	20
2.6.2.2 Chilling and freezing.....	21
2.6.2.3 pH and ionic strength	21
2.6.2.4 Emulsifier type and concentration	22
2.6.2.5 Dispersed phase	23
2.6.2.6 Aqueous/continuous phase.....	24
2.7 Emulsifying property of egg white protein	24
2.8 Improvement of egg white's emulsifying properties	25
2.8.1 Acid-induced treatment	25
2.8.2 Heat treatment.....	26
2.8.2 Maillard reaction.....	26
2.8.3 Phosphorylation reaction	27
2.8.4 Enzyme hydrolysis	27
2.8.4.1 Degree of hydrolysis (DH).....	28
2.8.4.2 Enzyme type and concentration	29
2.9 Commercial egg white products.....	30
2.9.1 Dried egg white powder	30
2.9.2 Frozen and liquid egg white	32
2.10 Conclusion of Literature review.....	33
Chapter 3. Effects of pH and heat treatment on EWL.....	34
3.1 Abstract	34
3.2 Introduction	34
3.3 Materials and Methods	36
3.3.1 Materials	36
3.3.2 Sample preparation	36
3.3.3 Zeta potential (ζ -potential) measurements.....	36
3.3.4 Turbidity measurement.....	37
3.4.5 Effect of heat treatment on EWL denaturation.....	37
3.4.6 Statistical analysis.....	38
3.5 Results and Discussion.....	38
3.5.1 Effect of pH on zeta potential of EWL solutions	38
3.5.2 Turbidity of EWL solutions.....	39
3.6 Effect of heat treatment on EWL	42

3.7 Conclusions	45
Chapter 4. Reduction of visible aggregates formed during emulsification using various methods	46
4.1 Abstract	46
4.2 Introduction	46
4.3 Materials and Methods	49
4.3.1 Materials	49
4.3.2 Effects of pre-homogenisation, heat treatment and oil concentration	49
4.3.2.1 Preparation of emulsions.....	49
4.3.2.2 Analysis of emulsion droplet size.....	51
4.3.2.3 Microscopic examination of emulsions	51
4.3.3 Effects of oil concentration and protein concentrations	51
4.3.3.1 Preparation of emulsions	51
4.3.3.2 Particle size and ζ -potential measurements	53
4.3.3.3 Creaming stability.....	53
4.3.3.4 Statistical analysis.....	53
4.4 Results and Discussion.....	53
4.4.1 Effects of pre-homogenisation, heat treatment and oil concentration on formation of EWP aggregates.....	53
4.4.1.1 Visual observations.....	53
4.4.1.2 Emulsion characteristics	56
4.4.2 Effect of oil and protein concentrations on formation of EWP aggregates during homogenisation.....	61
4.4.2.1 Visual observations.....	61
4.4.2.2 Particle size and ζ -potential of emulsions.....	63
4.4.2.3 Creaming stability.....	67
4.5 Conclusions	68
Chapter 5. Influence of protein concentration on stability of EWP emulsions: Effects of heat treatment, freezing and thawing, ionic strength and pH.....	70
5.1 Abstract	70
5.2 Introduction	71
5.3 Materials and Methods	73
5.3.1 Materials	73
5.3.2 Preparation of emulsions	73
5.3.3 Effects of environmental conditions on emulsions.....	74
5.3.4 Particle size and zeta (ζ) potential measurements	75

5.3.5 Confocal laser scanning microscopy	75
5.3.6 Statistical analysis.....	76
5.4 Results and Discussion.....	76
5.4.1 Effect of EWP concentration on particle size and size distribution	76
5.4.2 Effect of EWP concentration on ζ -potential	80
5.4.3 Effect of thermal treatment on protein concentration of EWP emulsions.....	80
5.4.3.1 Particle size	80
5.4.3.2 Confocal laser scanning microscopy	81
5.4.3 Effect of freezing and thawing	84
5.4.4 Effects of salt type and ionic strength	85
5.4.4.1 Particle size and size distribution.....	85
5.4.4.2 Emulsion stability	87
5.4.5 Effect of pH	89
5.5 Conclusions	91
Chapter 6. Emulsifying properties of EWP hydrolysates	93
6.1 Abstract	93
6.2 Introduction	94
6.3 Materials and Methods	96
6.3.1 Materials	96
6.3.2 Preparation of EWP hydrolysates.....	97
6.3.2.1 Effects of enzyme type, enzyme concentration and hydrolysis time on DH of EWPH.....	97
6.3.2.2 Effect of hydrolysis on emulsifying property of EWPH	98
6.3.3 DH	98
6.3.4 SDS-PAGE analysis.....	99
6.3.5 Preparation of emulsions	100
6.3.6 Particle size distribution.....	101
6.3.7 Effect of thermal treatment.....	102
6.3.8 Zeta potential (ζ -potential) measurements.....	102
6.3.9 Microscopic examinations.....	102
6.3.10 Colour measurement.....	102
6.3.11 Data analysis.....	103
6.4 Results and Discussion.....	103
6.4.1 Effect of enzyme concentration and hydrolysis time on DH of EWPH.....	103
6.4.2 Effect of enzyme type on DH of EWPH	105

6.4.3 SDS-PAGE analysis	106
6.4.4 Emulsifying properties of EWPH prepared with bromelain (4% E/S).....	110
6.4.4.1 Characteristics of emulsions	110
6.4.4.2 Particle size and size distribution of emulsions	112
6.4.4.3 ζ - potential of emulsions	115
6.4.4.4 Microscopic examination of emulsions	116
6.4.4.5 Colour of emulsions.....	117
6.4.4.6 Effect of heat treatment on EWPH emulsions	119
6.4.5 Emulsifying properties of EWPH prepared with 1% (w/w) bromelain and ficin	122
6.4.5.1 DH.....	122
6.4.5.2 Particle size and ζ -potential	122
6.4.5.3 Visual appearance and creaming stability.....	125
6.4.5.4 Microscopic examination of emulsion.....	126
6.4.5.5 Colour measurement	127
6.4.6 Characteristics of 0.3% FEWPH emulsions	128
6.5 Conclusions	130
Chapter 7. Overall Conclusions & Recommendations.....	131
References.....	135
Appendices.....	154

LIST OF TABLES

Table 2.1: Nutritional composition of egg white, whole egg and egg yolk.....	5
Table 2.2: Mineral and vitamin composition of egg white.....	5
Table 2.3: Chemical properties of egg white proteins	7
Table 2.4: Physicochemical properties of egg white proteins	8
Table 2.5: Metal complexes of ovotransferrin and their properties.....	10
Table 2.6: Applications of lysozyme in the food industry.....	12
Table 2.7: Functional properties of EWP in food systems	15
Table 2.8: Enzyme characteristics	29
 Table 3. 1: Heat treatment of egg white liquid (EWL) at different temperatures at different holding times.....	 37
 Table 4.1: Formulations of O/W UEWP emulsions and HEWP emulsions prepared with 1% w/w EWP and 4 different oil concentrations (5, 10, 15 and 20% w/w)	 50
Table 4.2: Formulations of O/W emulsions prepared with 1% (w/w) EWP and various oil concentrations (1, 3, 5, 6, 7 and 10% w/w).....	52
Table 4.3: Formulations of O/W emulsions prepared with 3% (w/w) EWP and various oil concentrations (1, 3, 5, 6, 7 and 10% w/w).....	52
Table 4.4: Mean particle size (D _{3,2}) and span values of UEWP and HEWP emulsions prepared with 1% w/w EWP and various oil concentrations (5, 10, 15 and 20% w/w oil)	56
Table 4.5: Mean particle size (D _{4,3} and D _{3,2}) and ζ-potential values of O/W emulsions containing different concentrations of canola oil (1,3, 5, 6, 7 and 10% w/w) stabilised by 1 and 3% (w/w) EWP	64
 Table 5.1: Addition of CaCl ₂ at different concentrations to EWP-stabilised emulsions containing 5% oil (w/w) and various protein concentrations (0.1, 0.3, 0.5, 0.8, 1.0 and 2.0% w/w)	 74
Table 5.2: Addition of NaCl at different concentrations to EWP-stabilised emulsions containing 5% oil (w/w) and various protein concentrations (0.1, 0.3, 0.5, 0.8, 1.0 and 2.0% w/w)	75
 Table 6.1: Enzyme characteristics	 97
Table 6.2: Hydrolysis conditions for preparing EWPH.....	98
Table 6.3: Formulations of emulsions with different types of EWPH	101

Table 6.4: Degree of hydrolysis (%) of EWP hydrolysates hydrolysed with three different enzymes using various E/S ratios (0.3, 0.5, 1, 2 and 4%). Hydrolysis was carried out at 50°C for 120 minutes with samples taken out at 0, 30, 60 and 120 minutes.	104
Table 6.5: Particle size and span values of emulsions prepared with various protein concentrations (1, 5 or 10% w/w) and 10% (w/w) oil concentration.	113
Table 6.6: Specifications of L, a, b colour values of FEWPH and BEWPH emulsions hydrolysed for 2 and 4 hours.	128
Table 6.7: Emulsion properties of emulsions prepared from 0.3% FEWPH containing 10% (w/w) oil and 1% EWP. Data are presented as mean and standard deviation (n =4)	129

LIST OF FIGURES

Figure 2.1: Structure of an egg showing the components of egg shell membrane, egg white layer and egg yolk. Adapted from Mine (2008).....	6
Figure 2.2: Schematic diagram of the L*a*b* tristimulus coordinate system for colour specification. Adapted from McClements (2015).....	19
Figure 2.3: Schematic diagram illustrating effect of emulsifier concentration on oil droplet coalescence and flocculation. Aadapted from Gao et al. (2017)	23
Figure 2.4: Schematic flowchart of production of commercial egg white products.....	32
Figure 3.1: ζ -potential of pH adjusted EWL at different protein concentrations (0.5, 1, 2, 3, 4, 5 and 10% w/w). Each data point is mean $\pm$ standard deviation of two independent measurements with triplicates (n=6).	38
Figure 3.2: Turbidity of EWL containing different EWP concentrations (0.5, 1, 2, 3, 4, 5 and 10%) at different pH ranging from pH 3 to 10. Each data point is mean $\pm$ SD for n=6.	41
Figure 3.3: Photographs of EWL solutions containing different EWP concentrations at pH levels ranging from pH 3 to 11.	42
Figure 3.4: Time taken for EWL solution at 20°C to reach desired temperatures (57, 58, 59, 60 and 62°C). Each data points represents mean $\pm$ SD (n=3).	43
Figure 3.5: Photographs of time dependent (0-19 minutes) changes in turbidity and denaturation of EWL (10% w/w protein) due to heat treatment (57, 58, 59, 60 and 62°C).	44
Figure 4.1: (a) Ultra Turrax (IKA T25 Basic, Staufen, Germany) and (b) 2-stage high pressure homogeniser (APV-2000 APV Manufacturing, Poland).....	50
Figure 4.2: Photographs showing insoluble aggregates in UEWP and HEWP emulsions prepared with 1% (w/w) EWP and different oil concentrations (5, 10, 15 and 20% w/w). UEWP and HEWP represents Unheated EWP Heat-treated EWP respectively.....	55
Figure 4.3: Particle size distributions of UEWP and HEWP emulsions prepared with 1% w/w EWP and different oil concentrations (5, 10, 15 and 20% w/w). UEWP and HEWP represents Un-heated EWP and Heat-treated EWP respectively.	59
Figure 4.4: CLSM images of EWP-stabilized emulsions at 5, 10 and 20% oil concentrations. UEWP: Un-heated egg white protein and HEWP: heat-treated egg white protein. Each bar notes represents 40 μ m.	60
Figure 4.5: Emulsions prepared with 1% (w/w) EWP and different oil concentrations (1-10%). Protein aggregates increased as oil concentration increased from 1 to 10% (w/w).	62
Figure 4.6: Emulsions prepared with 3% (w/w) EWP and different oil concentrations (1-10% w/w). Protein aggregates increased as oil concentration increased from 1 to 10% (w/w).	62
Figure 4.7: Photograph of droplets aggregates/flocs formed in the homogeniser during emulsion preparation.....	63

Figure 4.8: Particle size distributions of emulsions containing different oil concentrations (1, 3, 5, 6, 7 and 10% w/v) which were stabilized by 1% EWP (a) and 3% EWP (b)65

Figure 4.9: Creaming stability of emulsions stabilised containing different oil concentrations (1, 3, 5, 6, 7, and 10% w/w) stabilised by 1% EWP (a) and 3% EWP (b).68

Figure 5.1: (a) Z-average and (b) particle size distributions of O/W emulsions prepared with 5% (w/w) oil and various protein concentrations (0.1, 0.3, 0.5, 0.8, 1 and 2% w/w). Data points represents mean $\pm$ standard deviation (n=6). 77

Figure 5.2: ζ -potential of O/W emulsions prepared with 5% (w/w) oil and various protein concentrations (0.1, 0.3, 0.5, 0.8, 1 and 2% w/w). Data points represent mean $\pm$ standard deviation (n=6).80

Figure 5.3: Effects of heat treatment of temperature (60, 65, 70, 80 and 90°C) and holding time (0 and 30 minutes) on the mean particle size (Z-average) of emulsions prepared with different protein concentrations. Control indicates emulsion samples without heat treatment (a) 0.1% (b) 0.3% (c)0.5% (d) 0.8% (e)1% and (f)2%. Each data point indicates mean $\pm$ SD for n=6.82

Figure 5.4: Confocal images of O/W emulsions prepared with 5% w/v oil and various EWP concentrations (0.1, 0.3, 0.5, 0.8, 1 and 2% w/w) taken before and after heat treatment at 90°C for 0 or 30 minutes. NH=no heat treatment (i.e. control samples), H= heat treatment for 0 minute and H-30= heat treatment for 30 minutes. The scale bar inserted represents 20 μ m.83

Figure 5.5: Photographs of phase separation of emulsions stabilised by EWP containing various protein concentration (0.1, 0.3, 0.5, 0.8, 1.0 and 2.0% w/w) and 5% oil (w/w) after freezing at -20°C for 24 hours and thawing at 4°C and 20°C, respectively.84

Figure 5.6: Influence of (a) CaCl₂ (0, 5, 10, 50 and 100 mM) and (b) NaCl (0, 50, 100, 200, 400 and 600 mM) on the mean particle size (D_{3,2}) of O/W emulsions prepared with 5% oil (w/w) and various EWP concentrations (0.1, 0.3, 0.5, 0.8, 1.0 and 2.0% w/w). ...86

Figure 5.7: Photographs showing the influence of CaCl₂ concentration (0, 5, 10, 50 and 100 mM) on stability of emulsions containing 5% oil (w/w) and different EWP concentrations (0.1, 0.3, 0.5, 0.8, 1.0 and 2.0% w/w).88

Figure 5.8: Photographs showing the influence of NaCl concentration (0, 50, 100, 200, 400 and 600mM) on emulsions containing 5% oil (w/w) and different EWP concentrations (0.1, 0.3, 0.5, 0.8, 1.0 and 2.0% w/w).88

Figure 5.9: Influence of pH on ζ -potential of emulsion prepared with 1% (w/w) EWP and 5% (w/w) oil. Data represents mean $\pm$ standard deviation of two independent measurements with duplicates (n=6).90

Figure 5.10: Confocal images of pH adjusted (2, 4, 5, 7, 8.3, 9 and 10) of emulsions prepared with 1% (w/w) EWP and 5% (w/w) oil. The scale bar inserted represents 20 μ m.90

Figure 6.1: SDS-PAGE images of egg white protein hydrolysed with different types of enzymes (a) bromelain, (b) ficin and (c) papain using various amounts of enzymes (0.5, 1, 2 and 4% w/w). 107

Figure 6.2: SDS-PAGE images of egg white proteins hydrolysed with 2% (w/w) enzyme concentration of (a) bromelain (b) ficin and (c) papain at different time intervals (0, 30, 60 and 120 minutes).....	109
Figure 6.3: Photograph of OEWP and EWPH emulsions prepared with various EWP concentration (1, 5 and 10% w/w) and 10% (w/w) oil. (a) and (c) shows the presence or absence of droplet aggregates (b) and (d) shows presence or absence of phase separation. OEWP and EWPH represents original EWP and EWP hydrolysates, respectively.	111
Figure 6.4: Particle size distributions of emulsions prepared with various EWP concentrations (1, 5 and 10% w/w) and 10% oil concentration. (a) original egg white protein emulsion (b) egg white protein hydrolysate emulsion.....	114
Figure 6.5: Mean ζ potential of emulsions prepared with various protein concentrations and 10% (w/w) oil concentration. OEWP and EWPH represents original egg white protein and egg white protein hydrolysates respectively. Data points represent the means $\pm$ SD (n =6).	116
Figure 6.6: Microscopic images of control and hydrolysed emulsions prepared with various concentration of EWP (1, 5 and 10% w/w) and 10% (w/w) oil. OEWP and EWPH represents original egg white protein and egg white protein hydrolysates respectively. The scale bar inserted represents 20 μ m.	117
Figure 6.7: Colour specifications (L, a, b values) of emulsions prepared with original and hydrolysed egg white proteins. OEWP and EWPH represents original egg white protein and egg white protein hydrolysates respectively. Data points represent the means $\pm$ SD (n =6).	118
Figure 6.8: Photograph of thermal treatment of EWPH emulsions prepared with various EWP concentration (1, 5 and 10% w/w) and 10% (w/w) oil.....	120
Figure 6.9: Particle size diameter (D _{3,2}) of EWPH emulsions prepared with 1% (w/w) EWP and 10% (w/w) oil heat treated at various temperature (60, 65, 70, 80 and 90oC) for 0 and 30 minutes. Data points represent means $\pm$ SD (n =6).....	121
Figure 6.10: DH of egg white protein hydrolysates prepared with bromelain and ficin after 2 and 4 hours. Results are expressed as means $\pm$ SD of two independent replicates. ^{a,b} Means with different superscripts are significantly different (p < 0.05).	122
Figure 6.11: (a) Particle size diameter (D _{3,2}) and (b) ζ -potential of emulsions prepared with ficin and bromelain EWPH (at an E/S ratio of 1% w/w) hydrolysed for 2 and 4 hours containing 1% (w/w) EWP and 10% oil. FEWPH and BEWPH represents ficin egg white protein and bromelain egg white protein hydrolysates respectively.....	123
Figure 6.12: Particle size distribution of emulsions prepared with prepared with ficin and bromelain EWPH (at an E/S ratio of 1% w/w) hydrolysed for 2 and 4 hours containing 1% (w/w) EWP and 10% oil. FEWPH and BEWPH represents ficin egg white protein and bromelain egg white protein hydrolysates respectively.	125
Figure 6.13: Photograph showing appearance of FEWPH and BEWPH emulsions. B-2 = BEWPH after 2 hours, B-4= BEWPH after 4 hours, F-2= FEWPH after 2 hours, F-4= BEWPH after 4 hours.	126
Figure 6.14: Photograph showing the onset of phase separation appearance in FEWPH and BEWPH emulsions hydrolysed for 2 of 4 hours. B-2 = BEWPH after 2 hours, B-4= BEWPH after 4 hours, F-2= FEWPH after 2 hours, F-4= BEWPH after 4 hours.....	126

Figure 6.15: Microstructure of emulsions prepared with ficin and bromelain EWPH (at an E/S ratio of 1% w/w) hydrolysed for 2 and 4 hours containing 1% (w/w) EWP and 10% oil.	127
Figure 6.16: Photograph of 0.3% EWPH emulsions showing no separation on the day of preparation and phase separation the next day.	129

LIST OF APPENDICES

Appendix 1: Zeta potential for EWL solutions containing different protein concentrations (0.5-10% w/w) at different pH values using Zetasizer Nano ZS90	154
Appendix 2: Turbidity for EWL solutions containing different protein concentrations (0.5-10% w/w) at different pH values using Zetasizer Nano ZS90	155
Appendix 3: Time taken for EWL solution at 20°C to reach desired temperatures (57, 58, 59, 60 and 62°C).	156
Appendix 4: Z-average and Zeta potential of O/W emulsions prepared with 5% (w/w) oil and various protein concentrations (0.1, 0.3, 0.5, 0.8, 1 and 2% w/w).....	156
Appendix 5: Effects of heat treatment of temperature (60, 65, 70, 80 and 90°C) and holding time (0 and 30 minutes) on the mean particle size (Z-average (nm)) of emulsions prepared with different protein concentrations.	157
Appendix 6: Influence of CaCl ₂ (0, 5, 10, 50 and 100 mM) on the mean particle size (D _{3,2}) of O/W emulsions prepared with 5% oil (w/w) and various EWP concentrations (0.1, 0.3, 0.5, 0.8, 1.0 and 2.0% w/w).	158
Appendix 7: Influence of NaCl (0, 50, 100, 200, 400 and 600 mM) on the mean particle size (D _{3,2}) of O/W emulsions prepared with 5% oil (w/w) and various EWP concentrations (0.1, 0.3, 0.5, 0.8, 1.0 and 2.0% w/w).....	159
Appendix 8: Colour specifications (L, a, b values) of emulsions prepared with original and hydrolysed egg white proteins.	160

LIST OF SYMBOLS

$D_{4,3}$	Sauter mean diameter
$D_{3,2}$	Volume mean diameter
μm	micrometre
$^{\circ}\text{C}$	Degree Celsius
mM	Millimolar

LIST OF ABBREVIATIONS

AEP	Acid treated egg protein
AHEP	Acid-heat treated egg white protein
ANOVA	Analysis of variance
BEWPH-	Bromelain egg white protein hydrolysates
CaCl ₂	Calcium chloride
CLSM	Confocal Laser Scanning Method
Da	Dalton
DH	Degree of hydrolysis
DLS	Dynamic Light Scattering
E/S	Enzyme/Substrate
EW	Egg white protein
EWPH	Egg white protein hydrolysate
EW	Egg white
EWL	Egg white liquid
FEWPH	Ficin egg white protein hydrolysate
HCl	Hydrochloric acid
HEWP	Heat-treated egg white protein
HMW	High molecular weight
HPLC	High Performance Liquid Chromatography
kDa	Kilodalton
LMW	Low molecular weight
MPa	Mega pascal
NaCl	Sodium chloride
NaOH	Sodium hydroxide
nm	nanometre
OEWP	Original egg white protein
OPA	Ortho-phthalaldehyde
O/W	Oil-in-water emulsion
PDI	Polydispersity Index
pI	Isoelectric point
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SEM	Scanning Electron Microscopy
SLS	Static Light Scattering
TEM	Transmission Electron Microscopy
W/O	Water-in-oil emulsion
β -lg	Beta-lactoglobulin
ζ -potential	Zeta-potential

Chapter 1. Introduction

Egg white liquid (EWL) contains about 0.1% fat, 0.9% carbohydrate, 10% protein, 88% water and 0.5% ash (Li-Chan, 1995; Campbell, Raikos, & Euston, 2003; Hui & Al-Holy, 2007; Mine & Yang, 2007). Egg white protein (EWP) often called albumen present in EWL is a multi-functional food ingredient, widely known for its foaming, gelling, binding, adhesion and emulsifying properties because of its film-forming ability and amphoteric nature (Mine, 1995; Huntington & Stein, 2001; Arzeni, Perez, & Pilosof, 2012). These properties make EWP a desirable ingredient in meringues, ice cream, baked products and meat products (Mine, 1995; Chang et al., 2016). Additionally, apart from its unique and diverse functional properties, EWP possesses excellent nutritional value with a high amount of essential amino acids and high protein digestibility (Lomakina & Mikova, 2006; Raikos, Campbell, & Euston, 2007; Cho et al., 2014). Recent studies have also shown EWP to contain biological active components, such as antioxidant compounds with therapeutic potentials (e.g. immunomodulatory, antihypertensive, antithrombotic and antibacterial activity) (Davalos, Miguel, Bartolome, & López-Fandino, 2004; Mine, 2007; Miguel, Alonso, Salaices, Aleixandre, & López-Fandino, 2007; Murray & FitzGerald, 2007; Cho et al., 2014).

Despite EWP's incomparable gelling and foaming properties, it has a poor emulsifying potential when compared to other proteins (e.g. soy protein and whey protein) and egg yolk (Drakos & Kiosseoglou, 2006; Chang et al., 2016). Nevertheless, there are cholesterol-free salad dressings made with egg white which are sometimes found on the shelves of supermarkets, suggesting EWP could still be used as an emulsifying ingredient (Drakos & Kiosseoglou, 2006). The emulsifying ability of proteins is significantly determined by their surface hydrophobicity and surface net charge (Qian & McClements, 2011). The surface hydrophobicity is a protein's ability to adsorb to the oil phase of the interface between oil and water (Kim, Decker, & McClements, 2005). On the other hand, the surface net charge of proteins affects the diffusion rate of proteins through the interface (Delahaije, Wierenga, van Nieuwenhuijzen, Giuseppin, & Gruppen, 2013). Ovalbumin is one of the major egg white proteins imparting functionality as it constitutes around 54% of the total EWL protein (Mine, 1995; Mine, 2008; Belitz et al., 2009). Ovalbumin is unstable under alkaline and neutral conditions. Its hydrophobic amino acid residues are hidden in the interior of its

protein molecule with little exposed on the surface of the protein molecule. Thus, ovalbumin exhibits strong hydrophilic property with poor emulsifying capacity, restricting its application in non-acidic emulsion system.

Several studies have been carried out to improve and expand the emulsifying properties of EWP. Mine, Noutomi, & Haga (1991) reported that at pH 3.0, ovalbumin showed a high emulsifying ability due to an increase in flexibility and exposure of its hidden hydrophobic amino acids. Chang et al. (2016) demonstrated that acid treatment (AEP) and acid-heat treatment of EWP (AHEP) improved the emulsifying properties of EWP. Emulsions stabilized by AEP had a high zeta potential of +73 mV at pH 3.4, while at pH 4.2, emulsions stabilized by AEP and AHEP had zeta potentials of +43 mV and +49 mV, respectively. These treatments enabled sufficient protein to be adsorbed to the oil droplet surface with strong electrostatic repulsion to maintain emulsion stability. Kato, Ibrahim, Watanabe, Honma, & Kobayashi (1989) reported the use of heat treatment to improve the emulsifying ability of ovalbumin by investigating the effect of dry heat treatment of egg white (EW) at 80°C for 10 days. They reported a significant correlation between increased emulsifying capacity/stability and longer heating time. The use of heat treatment and extreme acidic conditions causes a protein to undergo partial or complete unfolding of its tertiary structure leading to an increase in its flexibility. This causes exposure of the buried hydrophobic amino acid groups of ovalbumin from its interior to its surface with increased flexibility and volume (Kato et al., 1989; Mine et al., 1991; Alizadeh-Pasdar & Li-Chan, 2000; Raikos, 2010; Chang et al., 2017).

Enzymatic hydrolysis is another way of improving the emulsifying ability of proteins. Hydrolysis can reduce the molecular weight of proteins and increase the amount of charge groups and the exposure of hidden reactive hydrophobic groups. This can increase the solubility of proteins over a wide pH range and induce a faster adsorption of small peptides to the interface for emulsion stabilization (Foegeding & Davis, 2011). Improved salt, thermal and emulsifying stability have been reported in egg white proteins (Cho et al., 2014; Chang et al., 2017); casein and whey proteins (Singh & Dalgleish, 1998; van der Ven, Gruppen, de Bont, & Voragen, 2001), soy (Jung, Murphy, & Johnson, 2005), milk (Agboola & Dalgleish, 1996) and rice bran (Thamnarathip, Jangchud, Jangchud, & Vardhanabhuti, 2016). However, the impact of enzymatic hydrolysis on the emulsifying properties of EWP has not been well researched.

The emulsifying properties of proteins are also significantly affected by physicochemical conditions, such as oil concentration, protein concentration, pH, type of salt and ionic strength which in turn affect the droplet size, polydispersity, rheology and stability of emulsions (Qian & McClements, 2011; Guo & Mu, 2011). A number of studies have investigated the effects of these factors on adsorption behaviour and emulsion stability of EWP (Padala, Williams, & Philips, 2009; Romero, Perez-Puyana, Marchal, Choplin, & Guerrero, 2017), whey (Osborne & Akoh, 2004; Sun & Gunasekaran, 2009), casein (Liang, Wong, Pham, & Tan, 2016), legume protein (Ettoumi, Chibane, & Romero, 2016) and sweet potato protein (Guo & Mu, 2011). Preliminary experiments carried out in this study on emulsions prepared with EWL showed the presence of large visible aggregates. The physical appearance was totally different from those prepared with other proteins like soy or milk proteins. Therefore, it is important to investigate factors causing aggregates and ways of reducing or completely removing it.

Therefore, the aims of this research project were;

1. To investigate the impact of pH and temperature on the properties of EWP (some factors such as protein concentration, turbidity and denaturation temperature were considered).
2. To explore the use of various factors, such as pre-heat treatment of EWL, oil concentration and protein concentration in reducing the formation of visible aggregates formed during emulsification.
3. To investigate the influence of protein concentration on the characteristics and formation of emulsions stabilised by egg white protein, and the effects of environmental conditions such as heat treatment, freezing and thawing, pH and ionic strength on the properties of the emulsions.
4. To evaluate the influence of enzyme type, enzyme concentration and hydrolysis time on degree of hydrolysis and emulsifying properties of egg white protein hydrolysates.

Chapter 2. Literature Review

The aim of this chapter is to review and provide an understanding on the nutritional, structure and physicochemical properties of egg white protein. Emulsion properties, emulsion destabilization mechanisms (e.g. coalescence, flocculation, phase separation and phase inversion) and some factors affecting emulsion stability (e.g. temperature, pH, ionic strength and chilling/freezing) are discussed. Some modifications used to improve the emulsifying properties of egg white protein as well as the production of commercial egg white products are also discussed in this review.

2.0 Egg white protein

Egg white remains as a popular, unique and widely used ingredient and functional food throughout the world due to its functional and nutritional properties of egg white protein (EWP) (Kato, Minaki, & Kobayashi, 1993; Van der Plancken, Van Loey, & Hendrickx, 2006; Brand & Kulozik 2015). This means that EWP is used extensively as a food ingredient in the food industry because its excellent multifunctional properties, such as gelling, emulsifying, foaming, binding and adhesion properties, meet most food formulation requirements (Mine, 1995; Hatta, Hagi, & Hirano, 1997; Huntington & Stein, 2001; Van der plancken et al., 2005, 2006). EWP is also used as an antimicrobial and antioxidant agent (Güçbilmez et al., 2007; Boyaci et al., 2016).

2.1 Nutritional composition of egg white

Egg white contains about 88% water, 10% protein, <1% carbohydrate, 0.1% fat, and 0.5% ash as shown in Table 2.1. Egg white is a rich source of protein but is very low in fat. Its major fatty acids are palmitic, arachidonic and stearic acids (Watkins et al., 2003). Its carbohydrate content is also low with about 50% of it in the form of free reducing sugars (mainly glucose) and the rest in conjugated form (Mine, 2002). EWP contains reasonable amounts of water-soluble vitamins but is deficient in fat-soluble vitamins (A, D, E and K) due to its aqueous nature (Campbell, Raikos & Euston, 2003; Belitz, Grosch & Schieberle, 2009; Mine & Zhang, 2013). They are also rich in minerals, such as potassium, sodium, phosphorous, magnesium, manganese, sulphur and chlorine (Watkins, 1995; Li-Chan & Kim, 2008; Johnson & Ridlen, 2016). Chlorine, sodium and potassium exist freely in the egg white medium, whereas calcium and magnesium are bound partly to proteins and

distributed heterogeneously between the thin and thick egg white layers (Sauveur, 1988; Li-Chan, 1995). The composition of vitamins and minerals is shown in Table 2.2.

Table 2.1: Nutritional composition of egg white, whole egg and egg yolk

Nutritional composition	Egg white (%)	Egg yolk (%)	Whole egg (%)
Water	88.6	49	74.4
Protein	9.7-10.6	15.7-16.6	12.8-13.4
Carbohydrate	0.4-0.9	0.2-1.0	0.3-1.0
Fat	0.1	34.5	11.9
Ash	0.5-0.6	1.1	0.8-1.0

Source: Hui & Al-Holy (2007) and Mine & Yang (2007)

Table 2.2: Mineral and vitamin composition of egg white

Mineral	(mg/100g)	Vitamin	(µg/100g)
Chloride	175	Thiamine (vitamin B1)	10
Sulphur	163	Riboflavin (vitamin B2)	430
Sodium	155	Niacin (vitamin B3)	90
Potassium	140	Pantothenic acid (vitamin B5)	250
Phosphate	18	Pyridoxine (vitamin B6)	10
Magnesium	10	Biotin (vitamin B8)	7
Calcium	8	Folic acid (vitamin B9)	12
Zinc	0.12	Cobalamin (vitamin B12)	0.1
Iron	0.1		
Copper	0.02		
Manganese	0.007		
Iodine	0.003		

Sources: Nys & Sauveur (2004), Huopalahti et al. (2007) & Belitz et al. (2009)

2.2 Structure and composition of egg white

Egg white as its name implies refers to the white part of hen's egg and is also called albumen (Mine & Yang, 2007; Belitz et al., 2009). It is an aqueous viscous solution constituting about 60% of the whole egg and contains the thin and thick egg white layers. It owes its viscosity and mucous nature to its high ovomucin protein content (Drakos & Kiosseoglou, 2006; Mine & Yang, 2007; Belitz et al., 2009). Ovomucin is a globular

heterogeneous protein and is present in four different layers of egg white (Li-Chan & Kim, 2008; Baron et al., 2016) as shown in Figure 2.1.

- The chalazae layer or inner thick layer (2.7%)
- The external thin liquid egg white (23.3%)
- The thick viscous egg white layer (57.3%) and
- The internal thin egg white layer (16.8%)

These proportions depend on egg size, laying rate, chicken breed and age, environmental conditions, health status of chicken, duration and method of egg storage (Sauyeur, 1988; Li-Chan et al., 1995).

COMPOSITION

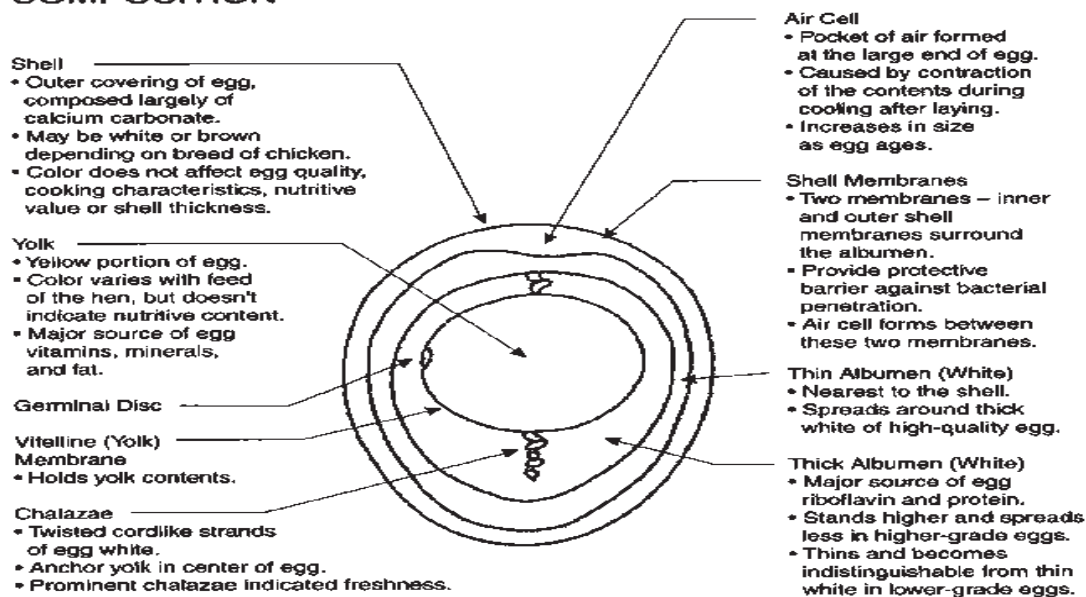


Figure 2.1: Structure of an egg showing the components of egg shell membrane, egg white layer and egg yolk. Adapted from Mine (2008).

2.3 Physicochemical properties of egg white

Egg white is a pseudoplastic liquid with a viscosity that depends on shear force. The pH of fresh albumen is about 7.6-7.9 and increases to 9.7 upon storage due to CO₂ diffusion through the egg shell. The pH increase also depends on time and temperature as it has been

shown that after 21 days of storage at 30-35°C, a pH of 9.4 was observed (Li-Chan, Powrie & Nakai, 1995). Egg white is a colloidal suspension of several proteins (9.7-10.6%) and most egg white proteins are glycoproteins (Drakos & Kiosseoglou, 2006). As already shown in Table 2.1, egg white contains about 10% protein. Studies have shown it contains over 40 different types of protein with major proteins including ovalbumin, ovotransferrin, ovomucin, lysozyme and ovomucoid and some minor proteins, such as avidin, ovoglobulins (G1 and G2), ovomucoid, cysteine, ovoflavoprotein and ovomacroglobulin (Stevens, 1991; Nys & Sauveur, 2004; Belitz et al., 2009). The physicochemical properties of these proteins are shown in Tables 2.3 and 2.4.

Table 2.3: Chemical properties of egg white proteins

Egg white protein	Amino acid residue	Disulphide bonds (S-S)	Free sulfhydryl (-SH)
Ovalbumin	385	1	4
Ovotransferrin	686	15	-
Ovomucoid	186	9	-
Ovomucin	872-2087	-	-
Lysozyme	129	4	-
Ovoglobulins	-	-	-
Ovoflavoprotein	-	2	-
Ovomucoid	-	21	-
Cystine	120	2	-
Ovostatin	-	4	-
Ovomacroglobulin	-	-	-
Avidin	-	-	-

Sources: Campbell et al. (2003), Wantabe et al. (2004), Belitz et al. (2009) & Baron et al. (2016)

Table 2.4: Physicochemical properties of egg white proteins

Egg white protein	% protein	MW ^a (kDa)	Pi	DT ^b (°C)	Characteristics
Ovalbumin	54	45	4.5-4.8	71.5-84	A phosphoglycoprotein, gelling and foaming property, denatures easily.
Ovotransferrin	13	77.7-80	6.1-6.6	57-61	Binds metallic ions and antimicrobial.
Ovomucoid	11	28	4.1	77	Inhibits trypsin, allergic reactions and heat stable in acidic condition.
Ovomucin	1.5-3.5	110	4.5-5.0	Heat stable	Heat stable, viscous, good foaming agent.
Lysozyme	3.4-3.5	14.3	10.5-11.0	75-81.5	Lyses gram negative bacteria.
Ovoglobulin G2	1	47	4.9-5.3	92.5	Good foam builder.
Ovoglobulin G3	1	50	4.8	-	Good foam builder.
Ovoinhibitor	1.5	49	5.1	69-72	Serine protease inhibitor.
Ovoflavoprotein	0.8	32-35	4.0-4.1	69-72	Binds riboflavin (vitamin B12).
Ovostatin	0.5	760-900	4.5-4.7	69-72	Glycoprotein.
Avidin	0.5	68.3	9.5-10	85	Binds biotin and antimicrobial.
Cystain	0.05	12.7	5.1	Heat stable	Protease inhibitor.
Ovoglycoprotein	1	24-24.4	3.9	69-72	-
Thiamine-binding protein		38	0	-	-
Glutamyl aminopeptidase		320	4.2	-	-

^a Molecular weight, ^b Denaturation temperature.

Sources: Stadelman & Cotterill (1995), Campbell et al. (2003) & Belitz et al. (2009)

2.4 Major egg white proteins and their characteristics

2.4.1 Ovalbumin

This is the major protein in egg white containing more than half of its protein (54%) (Mine, 1995; Mine, 2008; Belitz et al., 2009). Ovalbumin is a monomeric phospho-glycoprotein

containing carbohydrate and phosphate groups attached to its polypeptide chain (Belitz et al., 2009; Ahn, 2011). Resolution studies has shown it to be the only egg white protein containing a free sulfhydryl group (Table 2.1) (Nisbet, Saundry, Moir, Fothergill & Fothergill, 1981; Stein, Leslie, Finch & Carrell, 1991; Mine, 1995). Several studies however have reported there are three ovalbumin components; A1, A2, and A3 which differ in their phosphorous content as A1 has two phosphates per molecule; A2 1 and A3 none (Ternes, 2001; Belitz et al., 2009). It is known that ovalbumin is responsible for the gelling properties of egg white (Mine, 1995).

During storage ovalbumin is converted into a heat stable S-ovalbumin, though their amino acid composition remains the same (Lechevalier et al., 2007; Belitz et al., 2009). This conversion is due to pH rise as CO₂ is released through the pores of eggshell during storage and the conversion rate increases with high temperature, egg storage time and pH increase (Huang et al., 2011, 2012; Mine, 2015). Mine (2008) reported that this conversion posed a grave food processing challenge as high concentration of S-ovalbumin in egg produces heat-induced gels with poor strength. Huang et al. (2012) reported a loss of nutritional value in egg due to this conversion and stated that S-ovalbumin presence in egg can be used as an indicator of spoilage or freshness of egg. Studies have shown that the S-ovalbumin content in fresh egg increases from 5% to 81% in cold storage (2°C) for 6 months. This conversion has been attributed to thiol-disulphide exchange (Belitz et al., 2009).

2.4.2 Ovotransferrin (Conalbumin)

Ovotransferrin is a monomeric glycoprotein which belongs to a member of the transferrin family due to its binding to iron (Mine, 1995; Ahn, 2011; Wu & Acero-Lopez, 2012). This protein is reported to have a bilobal molecular structure. Studies have shown it to have a unique characteristic of binding to two molecules of metal ions (especially iron) at pH 6 and full dissociation of the metal complex occurs at pH <4; amino acids tyrosine and histidine are involved in the metal binding (Belitz et al., 2009). Table 2.5 shows the metal complexes of ovotransferrin with different properties according to types of metal ions complexed (Belitz et al., 2009). Ovotransferrin also has antimicrobial activity and can inhibit Gram negative and positive bacteria, fungi and viruses (Mine, 1995; Belitz et al., 2009; Ahn, 2011). Several studies have shown that ovotransferrin is the most heat susceptible egg white protein and has the lowest denaturation temperature of 60°C, coagulating to form a white milky gel at this low temperature (Belitz et al., 2009; Ahn, 2011). Superti et al. (2007)

suggested the use of ovotransferrin in iron-fortified food as a natural ingredient while Ahn (2011) suggested it to be used as a GRAS (generally recommended as safe) antimicrobial agent for meat and meat products since it is a cheap and natural antimicrobial like GRAS lactoferrin found in milk.

Table 2.5: Metal complexes of ovotransferrin and their properties

Metal ion	λ max (nm)	ϵ (1mol ⁻¹ cm ⁻¹)	Complex colour
Fe ³⁺	470	3280	Red/pinkish
Cu ²⁺	440	2500	Yellow
Mn ³⁺	429	4000	Yellow

Source: Belitz et al. (2009)

2.4.3 Ovomucin

Ovomucin is a highly viscous, gel-like sulphated, filamentous and fibre-like glycoprotein found in chalazae of egg white. It has been the subject of so many investigations due to its structural importance (Mine, 1995; Li Chan & Kim, 2008; Belitz et al., 2009; Strixner & Kulozik, 2011). Its ability to form fibrillar structures is responsible for egg white's viscosity and forms an insoluble water complex with lysozyme. This complex is associated with albumen thinning during egg storage and the dissociation of the complex is pH dependant (Mine, 1995; Li Chan & Kim, 2008; Belitz et al., 2009). It possesses characteristics, such as high carbohydrate content (33%), high molecular weight, heat stability and its unfractionated state containing 15% hexose, 2.6-8% sialic acid and 10-12% hexosamine. Foaming property, foam stability and emulsion capacity of albumen is attributed to it (Belitz et al., 2009; Omana, Wang & Wu, 2010; Mine, 2015).

Ovomucin is composed of two subunits; α -ovomucin (less soluble and carbohydrate poor with 2087 amino acid residues and a molecular weight of 230-250 kDa) and β -ovomucin (more soluble and carbohydrate rich with 870 amino acid residues and a molecular weight of 400-720 kDa) (Watanabe et al., 2004; Hiidenhovi, 2007; Hammershoj et al., 2008). In non-denaturing solvents and neutral pH, ovomucin is highly insoluble while it can be made soluble in mild alkaline condition using some mechanical (e.g. homogenisation, high pressure, sonication, etc.) and chemical treatments (e.g. reducing agents and denaturing solvents) (Belitz et al., 2009; Omana et al., 2010; Brand & Kulozik, 2016). Currently,

ovomucin's purification after isolation is still a challenge to scientists owing to its large molecular weight and poor solubility (Omana et al., 2010).

2.4.4 Lysozyme

Lysozyme is one of the oldest commercially used egg white proteins and has been studied widely by researchers (Mine, 1995; Juneja, Dwivedi, & Yan, 2012). It is a basic protein ubiquitous in nature as it can be also found in human secreted body fluids and tissues, plants, bacteria and bacteriophages as well as in egg white (Juneja et al., 2012). Egg white protein (EWP) is rich in lysozyme. Therefore, it is easily available, making it the commercial lysozyme source (Ahn, 2011; Juneja et al., 2012; Liburdi, Benucci & Esti, 2014). Its isoelectric point (pI 10.7) is higher than any other albumen protein which gives it the ability to bind to other egg white protein; ovalbumin, ovotransferrin, and ovomucin (Li-Chan & Kim, 2008; Ahn, 2011). Lysozyme has been shown to be the most soluble and stable egg white protein. It is stable on heating for 1-2 mins at 100°C and in acidic solution. Its disulphide bonds are responsible for its thermal stability (Ahn, 2011). It is also popularly known for its antimicrobial activity and is thus high demand in the food industry as a antimicrobial agent and preservative to extend the shelf life of meat, fish, milk, dairy, fruits and vegetables (Juneja et al., 2012; Liburdi et al., 2014; Erol et al., 2016) as shown in Table 2.6. It is also a good foam builder (Mine, 1995). However, lysozyme is found to be responsible for albumen thinning upon storage, which is due to its electrostatic interaction with ovomucin.

Table 2.6: Applications of lysozyme in the food industry

Product	Effects	References
Milk and dairy products		
Milk	Inhibition of <i>Listeria monocytogenes</i>	Tiwari et al. (2009) Charter & Lagarde (1999)
Hard cheese	Inhibition of <i>Clostridium tyrobutyricum</i> , <i>Bacillus cereus</i> Prevents late blowing, off flavours and accelerate ripening of cheese	Lopez-Pedemonte et al. (2003), Juneja et al. (2012)
Skim milk	Lysozyme, combined with high-pressure homogenization, affected the growth of <i>Listeria monocytogenes</i> and <i>L.</i> <i>plantarum</i>	Ahn (2011), Vannini et al. (2004)
Meat and fish		
	Lysozyme, combined with nisin, inhibited the growth of <i>Carnobacterium</i> sp.845 e	Nattress et al. (2001)
Minced meat	Lysozyme, combined with chitooligosaccharides, inhibited the growth of <i>Pseudomonas fluorescens</i> extending the product shelf-life	Rao et al. (2008)
Ham and bologna sausages	Lysozyme, combined with nisin, inhibited the growth of <i>Brochothrix</i> <i>thermosphacta</i> , <i>Leuconostoc</i> <i>Mesenteroides</i>	Gill & Holley (2000)
Pork loin	<i>Brachothrix thermosphacta</i> , lactic acid bacteria	Nattress & Baker (2003)
Raw minced tuna and salmon roe products	Control of <i>Listeria monocytogenes</i> growth	Tiwari et al. (2009), Juneja et al. (2012)
Beverages		
Red and white wine	Control the growth of <i>Oenococcus oeni</i> , <i>Lactobacillus spp.</i> , <i>Pediococcus</i> <i>damnosus</i> , <i>Pediococcus parvulus</i>	Isabel et al. (2009), Azzolini et al. (2010), Guzzo et al. (2011)
Beer	Growth delay of <i>Lactobacillus brevis</i> and <i>Pediococcus damnosus</i> Control lactic acid bacteria in beer	Makki & Durance (1996), Daeschel et al. (1999) Silvetti et al. (2010), Liburdi et al. (2014)
Fruit juice	Control of <i>Shigella Typhimurium</i> growth	Nakimbugwe et al. (2006), Raybaudi-Massilia et al. (2009)
Food packaging materials	Acts as an antimicrobial to inhibit Gram positive bacteria	Sebti et al. (2007), Del Nobile et al. (2009).

2.4.5 Ovomucoid

Ovomucoid is a member of the Kazal family of aprotase inhibitor. It is known best for its trypsin inhibitor and is more responsible for causing allergens more than any other albumen protein (Mine, 1995; Lechevalier et al., 2017b). It possesses characteristics, such as high water solubility, high heat stability and resistance to digestive enzymes (Hiidenhovi, 2007). Its peptide chain is comprised of three tandem homologous domains (I, II, III) with its putative active site (that inhibits serine proteases) found in domain II (Hiidenhovi, 2007; Belitz et al., 2009). Studies have shown no significant change in ovomucoid's chemical and physical properties when heated for a long time at 100°C under acidic conditions, though its biological activity is lost (Mine, 1995; Ahn, 2011). For ovomucoid sensitive patients, low ovomucoid egg white can be produced by chemical alteration to improve digestibility and reduce allergenicity using ethanol or acetone precipitation and heat treatment (Ahn, 2011).

2.4.6 Ovoflavoprotein

Ovoflavoprotein is the protein which was first characterized and isolated in egg white by Rhodes and his co-workers in 1959. It is a very heat stable protein with a denaturation temperature of 69-72°C and at pH 7 > 100°C, and it binds firmly to riboflavin and helps in the transportation of riboflavin from the blood serum to egg white (Belitz et al., 2009).

2.4.7 Ovoglobulin

Research has identified there are three globulins G1, G2, and G3 with G1 known as lysozyme. G2 and G3 have similar amino acid and carbohydrate contents. They are important as foaming agents in egg white. They become soluble and coagulate on heat treatment under mild saline solutions (Mine & Yang, 2007).

2.4.8 Ovoidin

Ovoidin makes up 1.5% of the total egg white protein content with a molecular weight of 50 kDa, its isoelectric point of pH 5.1-5.2 and denaturation temperature of 69-72°C. It is a serine protease (trypsin and chymotrypsin) inhibitor and also inhibits fungal and bacterial proteases (Stadelman & Cotterill, 1995; Campbell et al., 2003).

2.4.9 Avidin

Avidin is a strong basic protein representing 0.5% of the total egg white proteins. It binds firmly to biotin (B complex vitamin) and is also known as vitamin H. This glycoprotein

contains four subunits with each unit binding to four molecules of biotin. Its heat stability is very high (100°C for the protein and 120°C for avidin/biotin complex). Avidin is also known to have antibacterial activity and is used in diagnostic tests and immuno-assays (Belitz et al., 2009).

2.4.10 Cystatin

Cystatin contributes 0.05% of the total egg white protein with a molecular weight of 12,000 Da, its pI (isoelectric point) as pH 5.1 and denaturation temperature of 100°C at pH 4 and <100°C at pH 9. It is a cysteine proteinase (papain, ficin, dipeptidyl peptidase and cathepsin) inhibitor. Cystatin exists in two major forms, A and B, which are immunologically identical containing no carbohydrate but have different pI values of 6.5 and 5.6, respectively (Stevens, 1991; Mine, 1995).

2.5 Functional properties of egg white protein

Functionality in food systems refers to “any property aside from nutritional attributes that influences the effectiveness of an ingredient in food products” (Mine, 2002). A protein’s functional property is primarily associated with its chemical, physical and conformational characteristics (e.g. shape, amino acid composition, size, sequence, net charge and charge distribution) (Damodaran, 2005). Most functional properties of proteins as foaming, emulsifying and gelling agents depend on their ability to adsorb rapidly at the interface between oil (or air) and water phases (in case of emulsions or foams) and their interaction with each other to form gels (Li-Chan & Nakai, 1989; Chang et al., 2016; Arzeni, Pérez, & Pilosof, 2012).

As indicated above, EWP have multiple functional properties, such as foaming (e.g. meringues, baked goods), gelation (e.g. quiches, cakes), emulsification (e.g. batters, mayonnaise) and binding/adhesion (Kato et al., 1993; Mine, 2005; Van der Plancken et al., 2006). These properties are responsible for their extensive use in many different food products including formulated meat products, baked products, noodles, whipped products (Mine, 2005; Belitz et al., 2009; Liu, Jin, Lin, Jones & Chen, 2015). A summary of EWP functional properties is shown in Table 2.7.

Table 2.7: Functional properties of EWP in food systems

Functions	Mechanisms	Examples
Water binding	Hydrogen bonding and ionic hydration	Cakes and bread
Gelation	Water entrapment and immobilization, network formation	Gels, cakes, and bakeries
Cohesion, adhesion	Hydrophobic, ionic and hydrogen bonds	Pasta, baked goods
Emulsification	Adsorption and film formation at interphase	Cakes and dressings
Foaming	Adsorption and film formation at interphase	Whipped toppings, ice cream, cakes, desserts
Aroma-flavour binding	Hydrophobic bonds, entrapment	Low-fat bakery product, doughnuts

Source: Stadelman & Cotterill (1973), Hui & Al-Holy (2007).

2.6 Food emulsions

Emulsion property is an important functionality of food proteins. Emulsions are suspensions of two immiscible liquids, with one dispersed as droplets (dispersed phase) in the other (continuous phase) (Paraskevopoulou et al., 2007; Dickson, 2010). There are two major types of emulsion system; oil-in-water (O/W) and water-in-oil (W/O) emulsions. O/W emulsions consist of oil droplets dispersed in aqueous phase, e.g. milk, soups, sauces, dressings and mayonnaise. On the other hand, W/O emulsions consist of water droplets dispersed in oil phase, e.g. butter and margarine (Floury et al., 2004; Damodaran, 2005; Hui & Al-Holy, 2007). Multiple emulsion systems, such as oil-in-water-in-oil (O/W/O) and water-in-oil-in-water (W/O/W) emulsions, can also be prepared (McClements, 2010).

2.6.1 Food emulsion properties

Food emulsions are characterized by various physicochemical properties that depend on their composition and processing methods and conditions. In this section, emulsion properties, such as particle size, droplet charge, droplet microstructure and emulsion appearance, are discussed.

2.6.1.1 Droplet size and size distribution

The droplet size of an emulsion is an important emulsion characteristic that determines mouthfeel, appearance, rheology and stability (McClements & Rao, 2011). Food emulsions contain varying droplet sizes, it is therefore necessary to characterize emulsions, in terms of average size and particle size distribution. The particle size distribution of emulsion droplets is usually presented in a graph of droplet frequency (volume) against droplet size (diameter) (McClements, 2005; 2007). Changes in droplet size and distribution over time can be used in determining an emulsion stability (Friberg, Larsson & Sjoblom, 2004). Droplet size can be controlled by varying the emulsion composition (e.g. type and concentration of emulsifiers and oil, ratio of oil and water, etc.) and emulsification conditions (e.g. homogenization pressure and number of cycles) (McClements & Rao, 2011).

Static light scattering (SLS) and dynamic light scattering (DLS) are the most widely used method for measuring the particle size and size distribution of emulsions, depending on the size range of emulsion droplets. The SLS technique is used in measuring particle size ranging from 0.1 to 1000 μm but not suitable in measuring sizes below 100 nm (McClements, 2007). With this technique, particle size is measured by light scattering pattern produced by droplets when laser beam light passes through them. The Mie theory is used to predict the scattering intensities in particles that are homogenous and spherical in dilute suspensions (Horvath, 2009; Horne, 2011).

On the other hand, the DLS technique measures particle sizes ranging from 3 to 5000 nm (McClements, 2007). The measurement depends on the instrument optical arrangement, such as laser power, detection volume, measurement angle and attenuation (Malvern Instruments Limited, 2014). The DLS technique measurement is based on the intensity fluctuations of light scattered by the Brownian motion of droplet particles to produce a translational diffusion coefficient (Horne, 2011).

2.6.1.2 Droplet electrical charge

Droplets in an emulsion carry electric charges conferred to them by emulsifiers surrounding the surface of droplets. The droplet charge is characterized by zeta potential (ζ -potential) and is greatly influenced by emulsifier type and ionic strength of the continuous phase of emulsions. When oil droplets carry a high electrical net charge (e.g. $> \pm 30 \text{ mV}$), the

electrostatic interaction between the charged droplets becomes repulsive. This prevents oil droplets from coming close together, thereby providing stability against droplet aggregation. The surface net charge of droplets being net negative charges results from anionic emulsifiers used while a positively charged surface of droplets is due to an emulsion stabilized by cationic emulsifiers (Hasenhuettl, 2008). ζ -potential values are usually measured by electrophoresis and electroacoustic techniques with instruments where the electric charge is measured from electrophoretic mobility (McClements, 2005). These instruments use the DLS technique to measure the electrophoretic mobility (or particle velocity). Examples of such instrument include Zeta NanoSizer, Brookhaven Zeta PALS and Zetamaster ZEM5002 (Yin et al., 2009; Choi et al., 2010; Ray & Rousseau, 2013).

ζ -potential is formed between the electrical double layer of emulsion droplets and the continuous phase as the droplets move (Hunter, 1986). Generally, high electric charge usually $\geq \pm 30$ mV equates greater emulsion stability against aggregation and flocculation because of the strong electrostatic repulsion between the droplets (McClements, 2005; Chu et al., 2008). The magnitude of ζ -potential is decreased in the presence of minerals (e.g. Ca^{2+} or Na salts) and above a critical ionic strength, electrostatic repulsion between droplets is reduced causing emulsion instability (Hunter, 1986; McClements, 2005).

2.6.1.3 Droplet microstructure

The structural components (e.g. oil droplets, fat crystals, protein aggregates, surfactant micelles and gas bubbles) of an emulsion cannot be seen and detected readily and directly by the human eye. Therefore, a number of different microscopic techniques are used to obtain necessary information about the structure, organisation and dimensions of these components inside an emulsion. Microscopic techniques commonly used are optical microscopy, atomic force microscopy, electron microscopy and confocal laser scanning microscopy (CLSM) (McClements, 1999; Egerton, 2008; Murphy, 2012). Every microscopic technique has its own different principle and can examine different emulsion structures at different levels. Three qualities are define any type of microscopy: resolution magnification and contrast (Aguilera et al., 1999).

Optical microscopy is commonly used in studying emulsion microstructure for indirectly detecting an emulsion particle size distribution with relatively large droplets. It has a resolution limit of $0.2\ \mu\text{m}$ and cannot obtain reliable measurements when the size of

droplets is below 1 μm due to the design of its optical components (Murphy, 2012). The CLSM technique has a better resolution and produces much clearer images than the optical microscope, and often generates 3-dimensional images without the need to separate the structure of emulsions. The CLSM technique has the following functions: determining the size, aggregation state, droplet and particle location and concentration; monitoring the release of some ingredients in a delivery system; detection of location of emulsifiers and other ingredients (e.g. polysaccharides, phospholipids, or proteins) in the interface or surrounding phase (McClements, 2015). It is still limited in studying and observing small particles less than 200 nm.

Electron microscopy are used to study emulsion structural features smaller than the resolution lower limit of optical and CLSM techniques. It is mainly used for nanoemulsions and nanoparticles. Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) are examples of electron microscopy. These electron microscopic techniques generate images via electron beams to provide information about size, dimensions, concentration and location of components in a specimen. With TEM, 2-D images are produced by electron beams that have been transmitted through the samples, whereas with SEM 3-D images are generated when the electron beams scan across the surface of the specimen in a raster scan pattern (Dudkiewicz et al., 2011; Klang, Matsko, Valenta, & Hofer, 2012).

2.6.1.4 Emulsion appearance

A consumer's first impression of food emulsions is its appearance and it plays a key role in its perception and purchase (Caivano & del Pilar Buera 2012). The overall appearance of emulsion can be characterized by colour, surface gloss, opacity and visual homogeneity (Hutchings, 1999). This is determined by the interactions between the light rays from a visible region of electromagnetic spectrum and the emulsion (Clydesdale & Francis 1975; McClements 2002). The relationship between the composition and microstructure of emulsion and its appearance aids food technologists in designing improved emulsion quality (Wrolstad and Culver 2008). Food emulsion colours are affected by droplet size and concentration.

As it is difficult for the human eye to objectively identify precise object colour, the CIE (Commission International de l'Eclairage) $L^*a^*b^*$ is used as one of the most widely used

systems. This colour system uses the tristimulus coordinate concept in specifying colours using three coordinates: L^* , a^* , and b^* . As shown in Figure 2.2, L^* indicates lightness /darkness and its value ranges from 100 (white) to 0 (black); +ve a^* represents red coordinates and -ve a^* green coordinates and +ve b^* represents yellow coordinates and -ve b^* represents blue coordinates (McClements, 2015).

Figure 2.2: Schematic diagram of the $L^*a^*b^*$ tristimulus coordinate system for colour specification. Adapted from McClements (2015).

2.6.1.5 Rheological properties

Food emulsions are structurally and compositionally complex with varying rheological properties ranging from viscoelastic (yoghurt and desserts), fluid-like, dilute (milk, soft drinks), hard solids (refrigerated margarine and butter) or highly viscous (McClements & Rao, 2011; McClements, 2015). There is a direct relationship between the viscosity of an emulsion and the viscosity of the continuous phase. Modification made on the rheology of the continuous phase affects the entire emulsion rheology. An emulsion rheology depends on the structure, composition and droplet interaction (McClements & Rao, 2011). The Krieger-Dougherty equation (Equation 2.1) is used to describe the viscosity of a concentrated food emulsion (McClement, 1999).

$$\frac{\eta}{\eta_c} = \left(1 - \frac{\Phi}{\Phi_M}\right)^{-[\eta]q_M} \quad (2.1)$$

where η = viscosity of emulsion, η_c = viscosity of the continuous phase, Φ = volume fraction of dispersed phase, $[\eta]$ = intrinsic viscosity, for spherical particles it is 2.5, and Φ_M = maximum volume fraction of close packed droplets.

The Krieger-Dougherty equation shows there is an increase in viscosity of an emulsion when the oil phase volume fraction increases as particles are packed more closely. However, in a non-flocculated system, an increase in the oil volume fraction close to droplet concentration around 0.4 to 0.6 results in a viscoelastic, solid-like or plastic behaviour (McClements & Rao, 2011). Generally, the more viscous a continuous phase is, the slower the rate of aggregation and creaming of droplets. Thus, polysaccharides-stabilized emulsions are resistant against creaming or coalescence because of increased viscosity of the continuous phase, which slows the droplet movement and prevents them from coming together.

However, the increase in viscosity due to biopolymers depends on their molecular structure. Biopolymers with compact structures have lesser volume ratio than those with extended conformational structures. On the other hand, the volume ratio of linear and stiff polymers is higher than those of branched polymers (Walstra, 2003; McClements, 2005). The existence of biopolymers in the continuous phase provides increased viscosity, shear thinning behaviour and stability (McClements, 2005).

2.6.2 Factors influencing protein-stabilized emulsions

2.6.2.1 Thermal processing

In many applications, protein-stabilized emulsions are often subjected to thermal processing (pasteurization or sterilization) to increase their shelf life and consumer acceptance (Srinivasan, Singh, & Munro, 2003; McSweeney, Mulvihill, & O'Callaghan, 2004). Heat treatment may promote the production of smaller droplet by decreasing the interfacial tension between the aqueous and oil phases. However, globular proteins (e.g. egg white proteins or whey proteins) are sensitive to heat treatment above a critical temperature and lose their emulsion stabilizing ability (McClements, 2015). Thermal treatment causes globular proteins to unfold and aggregate exposing hidden reactive groups (e.g. sulfhydryl or non-polar groups) from their interiors. These reactive groups

subsequently increase the attractive interaction between the protein molecules leading to their aggregation and coalescence (Kim, Decker, & McClements, 2002; Singh, 2011; McClements, 2015). Heat treatment also affects the physical state of oil droplets (solid or liquid) and influences the rheology and stability of emulsions.

A comparison on the thermal stability of emulsions stabilized by acid-treated egg white and acid-heat induced egg white was investigated by Chang et al. (2016). They reported thermal stability also depended on pH as droplets were stable against extensive aggregation at lower pH after heating (90°C for 30 minutes). They also reported that synergistic application of acid and heat conferred better stability than just acid treatment. This treatment promoted transition of the tertiary structure resulting in strong electrostatic repulsion between the charged droplets. Whey proteins was also reported to have better thermal stability against protein aggregation at acidic pH than at neutral pH (GoTo, Calciano, & Fink, 1990).

2.6.2.2 Chilling and freezing

Most food products are usually frozen or chilled during storage and then thawed or warmed up before use. Reduced temperature storage can extend the shelf-life of food products for a long time by reducing chemical, microbial or enzymatic reactions. However, many O/W emulsions are unstable after thawing which undergo extensive coalescence and phase inversion. Many factors have been attributed to these instability; biopolymer conformation changes, ice formation, freeze concentration, fat crystallization, and interfacial phase transition (McClements, 2004). Partial coalescence can occur when fat crystals of crystalline droplet pierce into another crystalline droplet resulting in irregular shaped aggregates and reduced creaming stability. Such emulsions result in thickening of cream which is considered as an undesirable attribute (Vanapali, Palanuwech, & Coupland, 2002). On the other hand, ice crystallization can reduce the availability of free water needed for the hydration of emulsifiers; increase droplet-droplet interactions; penetrate oil droplets to rupture their interfacial structure; cause emulsifiers to lose their effectiveness; and increase ionic strength encouraging screening of electrostatic repulsion between droplets (McClements, 2004).

2.6.2.3 pH and ionic strength

Protein-stabilized emulsions are susceptible to destabilization due to changes in pH and ionic strength. At pH near their isoelectric point the electrical charges on the droplets of

emulsion become neutralized, thus the electrostatic repulsive force between droplets becomes weakened causing droplet flocculation and aggregation (Kulmyrzaev & Schubert, 2004). However, in extreme acidic conditions, the emulsifying ability of egg white proteins is increased because hydrophobic groups on the surface of egg white protein reduce the kinetic barrier for adsorption at the interface (Alizadeh-Pasdar & Li-Chan, 2000). Flocculation and aggregation due to salt addition occur when the ionic strength exceeds a certain critical level. The level of electrostatic interactions between droplets are determined by the mineral type and concentration present in the continuous phase via different mechanisms, such as ion binding, ion bridging, electrostatic screening and water structure. Generally, high ionic strength increases interactions between droplets due to screening of charges and reduction of the electrical double layer (Hunt & Dalgleish, 1995; McClements, 2004). For some emulsions, the order in which the salts are added (before or after homogenisation or heat treatment) affects emulsion stability against droplet aggregation (Kim et al., 2005).

2.6.2.4 Emulsifier type and concentration

A type of emulsifier used is an important factor that determines an emulsion stability. The two major functions of an emulsifier are (i) to reduce the interfacial tension between the aqueous and oil phases and, (ii) to form a thick protective layer around the droplets that prevents droplets coming together. For example, protein-stabilized emulsion is susceptible to pH and ionic strength changes because their stabilization is based on electrostatic repulsion. On the other hand, polysaccharide and non-ionic surfactants-stabilized emulsions are not sensitive to changes in pH and ionic strength since the emulsion stabilization is mainly by steric repulsion (Qian, Decker, Xiao, & McClements, 2011). However, the efficiency of an emulsifier is also influenced by other factors, such as (i) the ratio of emulsifier to dispersed phase, (ii) the time it takes an emulsifier to move from bulk phase to droplet surface, (iii) the amount of emulsifier needed to reduce the interfacial tension between oil and aqueous phases, (iv) the emulsifier extent at changing the interfacial rheology, and (v) the emulsifier effectiveness in generating a protective layer around the droplets against aggregation and coalescence (McClements, 2015).

The influence of emulsifier concentration on mean particle can be divided into two categories: (i) insufficient emulsifier: this happens when the emulsifier concentration is deficient and not enough to cover the newly formed droplets surface. As a result, gaps are

produced in the interfacial membranes surrounding the oil droplets leading to the droplets coming together to form droplet coalescence (McClements, 2004), and (ii) excess emulsifier: this happens when there is more than enough emulsifier to completely cover the surface of the newly formed droplet. The causes unadsorbed proteins to act as bridges among themselves and the oil droplets resulting in large flocs formation that enables bridging flocculation (Ettoumi, Chibane, & Romero, 2016) as seen in Figure 2.3.

Figure 2.3: Schematic diagram illustrating effect of emulsifier concentration on oil droplet coalescence and flocculation. Aadapted from Gao et al. (2017)

Several researchers have investigated the effect of protein concentration on droplet size and emulsion stability. Padala, Williams, & Philips (2009) investigated the effect of various EWP concentrations (0.1, 0.2, 0.3, 0.5, 0.75 and 1% w/w) with 20% limonene oil at pH 3.5 and 7.5. Romero et al. (2017) prepared EWP using various EWP concentrations (0.75, 1.5, 3.0 and 5.0%) containing 65% sunflower oil. They reported decreased droplet size with increasing EWP concentration. Similar results have been reported with other protein sources: whey proteins (Hebishy, Buffa, Guamis, Biasco-Moreno, & Trujillo, 2015; Lizarraga, Pan, Anon, & Santiago, 2008); soy proteins (Palazolo, Sobral, & Wagner, 2011); and sweet potato protein (Guo & Mu, 2011). They attributed these decreases to increase viscosity in the continuous phase which reduced the movement of the droplets and droplet diffusion within the emulsion (Jafari, Beheshti, & Assadpoor, 2012).

2.6.2.5 Dispersed phase

In an O/W emulsion, the disperse phase is oil or fat dispersed in an aqueous continuous phase. The type and volume of oil affects an emulsion characteristics, such as flavour and aroma as some oils such as lemon oil contain flavour compounds; colour and opacity; viscosity as an emulsion viscosity increases as oil concentration increases (McClements,

2005). It also determines the degree of crystallization of oil/fat droplets which in turn affects the physical stability, texture, mouthfeel and appearance of food emulsions (McClements, 2015).

2.6.2.6 Aqueous/continuous phase

The aqueous phase of O/W emulsions is made up of water. However, emulsion properties and stability are affected by the presence of components (e.g. emulsifier, salt, etc) in the continuous phase (Norde, 2003; Sosa-Herrera, Berli, Martinez-Padilla, 2008). Factors, such as dielectric constant, pH, ionic strength and viscosity, affect the continuous phase and emulsion stability. As the continuous becomes more viscous, smaller droplets can be produced during homogenisation and the increased viscosity can also prevent oil droplets from coming into contact (McClements, 2005).

2.7 Emulsifying property of egg white protein

EWP are popularly known as effective gelling (Eleya & Gunasekaran, 2002; Weijers, van der Velde, Stijnman, van der Pijpekamp, & Visschers, 2006; Alleoni, 2006; Tomczynska-Mleko et al., 2016) and foaming agents (Mine, 1995; Pernell, Foegeding, Luck, & Davis, 2002; Raikos, Campbell, Euston, 2007; Altalhi, 2013) because of their ability to form films and amphoteric nature (Huntington & Stein, 2000). However, its emulsifying property is considered poor when compared to other emulsifiers like soy protein, whey protein or egg yolk (Drakos & Kiosseoglou, 2006; Chang et al., 2016).

Physicochemical properties, such as surface net charge and surface hydrophobicity, are key factors in determining the emulsifying ability of a protein (Qian & McClements, 2011). The surface hydrophobicity influences the ability of a protein to adsorb to the lipid side of the interface (Kato & Nakai, 1980; Kim, Decker & McClements, 2005; Chang et al., 2016). On the other hand, the surface net charge influences the rate of protein diffusion to the interface and affects protein solubility in the aqueous phase (Delahaije, Wierenga, van Nieuwenhuijzen, Giuseppin, & Gruppen, 2013).

Ovalbumin which is the key component of EWP contains about 385 amino acids with one third of it charged, half hydrophobic and the majority acidic (giving it a pI of 4.5) (Huntington & Stein, 2001). Most of its hydrophobic amino acid residues are hidden and buried deep in the molecular structure and very few are exposed for emulsification to take

place. This is responsible for EWP's strong hydrophilic properties and poor emulsifying property especially in alkaline and neutral conditions (Drakos & Kiosseoglou, 2006; Chang et al 2016; Niu et al., 2016).

2.8 Improvement of egg white's emulsifying properties

The limited application of EWP as emulsifiers in food systems has led several researchers in investigating methods and techniques for improving the functional property of EWP for consumption and target use in the food industry (Mine, 2014). Treatments, such as acid treatment, heat treatment, Maillard reaction, phosphorylation and enzymatic hydrolysis, are discussed below.

2.8.1 Acid-induced treatment

At neutral and alkaline pH, emulsions stabilised by EWP are very unstable restricting their application in non-acid emulsion system. Previous studies have reported good emulsifying ability and stability of ovalbumin under acidic conditions (Mine, Noutomi, & Haga, 1991). They reported high emulsifying ability of ovalbumin at pH 3 correlating to its increased surface hydrophobicity and flexibility. Chang et al. (2016) investigated the effect of acid treatment at pH 3.0-4.2 on the hydrophobicity, ζ -potential and emulsifying properties of EWP. Their results indicated better emulsifying property, heat and salt stability of acid treated EWP due to greater hydrophobicity and greater net charge. They also reported a very high ζ -potential value of 73 mV at pH 3.4 which promoted emulsion stability against droplet aggregation and phase separation over a 3 weeks storage period.

Significant improvement in the functional property of soy proteins after acid or alkaline treatment owing to higher net charge and surface hydrophobicity have been reported (Jiang, Chen, & Xiong, 2009). Whey proteins have also showed better stability at acidic pH than at neutral pH (GoTo, Calciano, & Frank, 1990). At extremely acidic conditions, the hidden hydrophobic groups of globular proteins are exposed and undergo partial or complete unfolding, become more flexible and yet still retaining its structure (Raghavan, & Kristinsson, 2007). Owing to more exposure of the hydrophobic groups to the surface, there is reduction of the kinetic barrier to enable ovalbumin adsorption to the interface (Alizadeh-Pasdar & Li-Chan, 2000).

2.8.2 Heat treatment

Heat treatment can also contribute to improvement of emulsifying properties of ovalbumin by significantly increasing its hydrophobicity. Heat denaturation causes changes in a protein's secondary and tertiary structure resulting in further negatively charged acidic amino acid residues exposed to the surface (Chang et al., 2016). Heat treatments also lead to complete or partial unfolding of the protein's tertiary structure to expose the buried hydrophobic amino acid groups and its conformations are exposed to increase its flexibility and excluded volume in the case of globular proteins like EWP (Kato, Osako, Matsudomi, & Kobayashi, 1983; Raikos, 2010). Although egg white emulsion formation and stabilization can be enhanced by extreme pH change and pre-heat treatment, egg white has poor thermal stability limiting heat modifications of their functional properties.

Chang et al (2016) suggested the use of acid (pH 3.0) and moderate heat (60°C for 15 mins) treatment on EWP owing to their mild effect and induction of its tertiary structure transition with little changes in its secondary structure. In addition, they reported that this method could be very efficient in bringing about partial expansion of EWP and avoiding aggregate formation. They reported that egg white emulsions prepared at pH 3.0-4.2 using acid and acid-heat treatment had a zeta potential of 43-49 mV indicating sufficient protein adsorption to the oil droplet surface and emulsion stability against flocculation or aggregation. They concluded that acid or acid-heat treated EWP showed greater hydrophobicity and higher net charge than untreated EWP.

2.8.2 Maillard reaction

Maillard reaction has been extensively used to improve the emulsification of egg white by forming protein-sugar or polysaccharide conjugates (Campbell et al., 2003; Nakamura & Kato, 2000). This is attributed to the attachment of a hydrophilic polysaccharide to the egg white protein chain which causes the Maillard conjugates to adsorb better at the lipid-water phase resulting in the formation of a stable emulsion since the hydrophobic side chains are attached to the oil phase and the hydrophilic group to the water phase (Campbell et al., 2003). Conjugation of ovalbumin with galactomannan (e.g. guar gum) through Maillard reaction improved the emulsifying ability of ovalbumin by 15% at pH 7.4 and was more effective as an emulsifier than a commercial emulsifier, such as sucrose fatty acid ester, in acidic pH and salt conditions (Nakamura & Kato, 2000). The results were consistent with the results of Kato et al. (1993) as they reported increased emulsifying activity and stability

with the use of galactomannan conjugated egg white protein formed after dry heating when compared to native egg white protein. Campbell et al. (2000) indicated that Maillard conjugates mimicked egg yolk lipoprotein, hence their improved emulsifying capacity and stability. Another study reported enhanced emulsifying property of ovalbumin conjugate with glucuronic acid which was 3.2 times effective than untreated ovalbumin (Aoki et al., 1999).

2.8.3 Phosphorylation reaction

Phosphorylation is another efficient method confirmed to increase the functionality (e.g. foaming, gelation, heat stability, water/oil interface binding capacity, etc.) of food proteins like egg white proteins, milk proteins and soy proteins (Nayak, Arora, Sindhu, & Sangwan, 2006; Miedzianka & Peksa, 2013). The foaming property of phosphorylated EWP was significantly higher than that of native EWP (Hayashi, Nagano, Enomoto, Li, Sugimoto, 2009). Also, surface binding, emulsifying properties, heat-induced insolubility, and calcium phosphate-solubilizing ability of ovalbumin were increased by phosphorylation (Lv & Chi, 2012). Xiong, Zhang, Ma (2016) phosphorylated ovalbumin with sodium tripolyphosphate and reported increased zeta potential and smaller particle size with uniform, dense droplet size distribution compared to untreated ovalbumin. They concluded that phosphorylation introduced a negative charged phosphate group which caused a strong electrostatic repulsion between oil droplets. This resulted in improved steric stabilization, prevention of aggregation and coalescence of droplets, reduced droplet size and increased emulsifying capacity.

2.8.4 Enzyme hydrolysis

Enzymatic hydrolysis of proteins has been studied extensively by several researchers over the last 60 years (Aldler-Nissen, 1986; Lahl & Braun, 1994). It is regarded as the breakdown of proteins into free amino acids and smaller peptides. Enzymatic proteolysis of proteins is an important bioprocess for modifying the physical, chemical, nutritional and functional of intact proteins (Kristinsson & Rasco, 2000; El-Salam & El-Shibiny, 2017). Different protein sources, such as egg white (Cho et al., 2014; Chang et al., 2017), whey (Singh & Dalgleish, 1998; van der Ven et al., 2001), soy (Wu, Hettiarachchy, & Qi, 1998; Jung, Murphy, & Johnson, 2005; Chen, Chen, Ren, & Zhao, 2011), milk (Agboola & Dalgleish, 1996) and rice bran (Thamnarathip, Jangchud, Jangchud, & Vardhanabhuti,

2016), have been investigated for the effect of enzymatic hydrolysis on their emulsion forming and stabilising abilities.

Enzymatic hydrolysis of protein has shown to decrease its average molecular weight, improve its solubility over a wide pH range, increase its surface hydrophobicity and increase the number of charged groups which enable better adsorption of the protein to the droplet surface (Tsumura, 2009; Foegeding & Davis, 2011). Singh & Dalgleish (1998) reported better heat stability of hydrolysed whey protein emulsion compared to original whey protein emulsion. Thamnarathip et al. (2016) also reported improved heat and emulsifying stability of rice bran hydrolysate emulsion. On the other hand, Chang et al. (2017) observed improved thermal and salt stability and smaller droplet size in hydrolysed egg white protein emulsion. They attributed the improved ability to stronger repulsive force between droplets. Several factors such as degree of hydrolysis (DH) (which influences molecular weight and peptide length) and hydrolysis conditions (e.g. temperature, time, enzyme type and concentration), affect the emulsifying properties of protein hydrolysates (Lamsal et al., 2007).

2.8.4.1 Degree of hydrolysis (DH)

DH is a widely-used indicator for comparing proteolytic hydrolysis process. It measures the degree of a protein's hydrolytic degradation (Noh & Suh, 2015) and influences the molecular size and amino acid composition which all affect emulsifying ability (Kristinsson & Rasco, 2000). Nevertheless, very high DH (extensive protein hydrolysis) is detrimental to protein's emulsifying ability because of the short chain peptides being formed which saturate the continuous phase instead of adsorbing to the oil-water interface (Agboola, Singh, Munro, Dalgleish, & Singh, 1998; Conde & Patino, 2007). Turgeon, Gauthier, & Paquin. (1991) pointed out that even though small peptides rapidly diffuse and adsorb onto the oil-water interface, their efficiency in decreasing the interfacial tension is less as the peptides don't unfold and re-arrange at the interface. In a comparative study of emulsifying property of whey protein hydrolysates with different DH levels, extreme DH levels (>20%) produced short term stability due to rapid droplet aggregation, coalescence and oiling off occurred (Scherze & Muschiolik, 2001). Another study investigated the emulsifying property of peanut protein hydrolysate using Alcalase at varying DH levels (10, 20, 30 and 40%) and reported decreased emulsifying property at DH >10% (Jamdar et al., 2010). Emulsions prepared with wheat gluten hydrolysates showed remarkable emulsifying

property at 5% DH (Kong, 2007). In summary, higher emulsifying ability is obtained at lower DH levels and long peptide units.

2.8.4.2 Enzyme type and concentration

Commercial enzymes used by researchers for proteolytic process can be of plant origins like papain (Chen, Chang, Wang, & Cheng, 2009; Cho et al., 2014), bromelain and ficin (Cho et al., 2014; Noh & Suh, 2015) or from microbial sources (alcalase, flavourzyme, neutrase, protamex and protease N) (Chen et al., 2009; Cho et al., 2014; Noh & Suh, 2015) and also digestive proteases (e.g. trypsin, pepsin and chymotrypsin) (Davalos, Miguel, Bartolome, & Lopez-Fandino, 2004; Chen et al., 2009). Hydrolysis characteristics of major enzymes are shown in Table 2.8. In enzymatic hydrolysis, the choice of substrate and enzyme specificity determines the protein's degree of hydrolysis, molecular size, hydrophobic/hydrophilic balance and hydrolysate functionality; the broader the enzyme specificity, the more complex the peptide profile and the smaller the peptides are (Tavano, 2013).

Table 2.8: Enzyme characteristics

Enzyme	Source	Type	Optimum conditions	
			Temperature (°C)	pH
Alcalase	<i>Bacillus licheniformis</i>	Endopeptidase	50-60	8.0-9.0
Bromelain	<i>Ananas comosus</i>	Endopeptidase	40-65	4.0-9.0
Ficin	<i>Ficus carica</i>	Endopeptidase	45-50	5.0-6.0
Flavourzyme	<i>Aspergillus oryzae</i>	Complex	45-50	5.0-7.0
Neutrase	<i>B. amyloliquefaciens</i>	Endopeptidase	45	6.0-7.0
Papain	<i>Carica papaya</i>	Endopeptidase	50-70	5.0-7.5
Protamex	<i>Bacillus</i> sp.	Complex	35-60	5.5-7.5

Source: Cho et al. (2014) and Noh & Suh (2015)

The issue of selecting the best enzyme for proteolytic degradation of EWP has been investigated by several researchers. Noh & Suh (2015) hydrolysed egg white liquid using several proteases (alcalase, collupulin, ficin, flavourzyme, neutrase and protamex) and

reported a highest DH and amino-nitrogen content with alcalase compared to other enzymes, followed by neutrase. Higher DH levels signify more hydrolysed EWP (Cho et al., 2014; Noh & Suh, 2015). Another study by Cho et al. (2014) reported neutrase with the highest DH and amino-nitrogen content after hydrolysing egg white powder with the same enzymes used by Noh & Suh, (2015). However, highest DH was reported with papain after hydrolysis of duck egg white liquid (Chen et al., 2009).

Enzymes contribute to the major expense of the hydrolytic process. It is therefore necessary to determine the right quantity of enzymes needed to hydrolyse proteins effectively. This prevents waste of enzymes, minimises enzyme use and reduces enzyme cost. Several researchers have investigated the effect of enzyme concentration on DH (Wu, Wang, & Xu, 2008; Chen et al., 2009; Noh & Suh, 2015). They all reported increased hydrolysis with increasing enzyme concentration but suggested use of enzyme concentration with similar degree of hydrolysis. Chen et al. (2009) hydrolysed duck egg white with 1 and 2% E/S papain and reported no change in DH levels and number of peptides after 1 hour.

2.9 Commercial egg white products

Commercially, egg white can be found in different physical forms (e.g. dried, liquid and frozen) which are used in the food industry for their applications as ingredients in manufacturing many different products, such as confectioneries (marshmallows), baked goods (cakes), pastry products, meat products, ice cream, whipped cream, soup powders and salad dressings (Belitz et al., 2009). They are also used as natural food preservatives to control microbial growth in wine, kimchi, pickles, sushi, cheese and Chinese noodle (Mine et al., 2004; Erol et al., 2016). Figure 2.4 shows a schematic presentation of the production of egg white products.

2.9.1 Dried egg white powder

Dehydration process is used to preserve eggs by moisture removal to halt microbial growth and reduce the rate of chemical reactions (Mine & Yang, 2010). Dried egg white has the advantage of easy handling, low storage and transportation cost, better uniformity, extended shelf life and specific functional properties (Lechevalier, Jeantet, Arhalias, Legrand & Nau, 2007; Mine & Yang, 2010). This enables its use in convenience foods like salad dressing, ice cream, bakery mixes, bakery foods, confectioneries, mayonnaise and pasta (Mine & Yang, 2010). The type of drying process of dried egg white depends on the required

functional properties. The popular methods are spray drying non-whipping and spray drying-whipping (sodium lauryl sulfate is the whipping aid). Spray drying-whipping is used mainly in angel and layer cake (Bergquist, Lorimor, & Wildy, 1992). The dried egg white exists in three major forms, such as powder, flakes and granules. In specific applications, spray-dried egg white particles form undesirable clumps on water addition. To resolve this, instant egg whites are used for rapid dispersal and dissolving of the particles on water addition (Bergquist, et al., 1992).

The production of dried egg white starts with egg storage for 2 days at 15°C to enable easy separation of its components. In some countries, before breaking open, the eggs are disinfected with an aqueous chlorine solution (200 mg/l) (Belitz et al., 2009). After separation, the liquid egg white is purified then pasteurised. Egg white requires a low pasteurization temperature of 52°C for 7 minutes since it coagulates around 55 to 57°C. However, it depends on a combination of temperature and heating time. The sugars in egg white are removed after pasteurization and before the spray drying process to prevent Maillard reaction from occurring which can result in undesirable aroma and brown discolouration (Stadelmann & Cotterill, 1977; Lechevalier et al., 2007; Belitz et al., 2009).

The sugar is removed by microbial sugar fermentation, where the pasteurized liquid egg white's pH (9.0-9.3) is adjusted to pH 7.0-7.5 using lactic or citric acid. It is then inoculated with *Streptococcus spp.* or *Aerobacter spp.* at 30-33°C (Belitz et al., 2009). In egg white drying, spray drying is the most important process. The liquid egg white is concentrated from 11-18% solids by membrane filtration (at 45-50°C) which is an energy saver in the drying process. After this, egg white is dried in a high-pressure dispersion air stream of 165°C which allows it to be heated up to 50-60°C. The product finally undergoes post-pasteurization (dry heating) in a heating room for a minimum of 7 days at 55°C to kill spoilage and pathogenic microorganisms like *Salmonella spp.* It also improves the foaming and gelling properties of egg white (Kato et al., 1989; Mine, 1996; Handa et al., 2001). The dried egg white powder has an unlimited shelf life. It has a maximum moisture content of 8.0%, minimum fat of 0.12%, protein 80% and ash 5.7% (Stadelmann & Cotterill, 1977; Belitz et al., 2009).

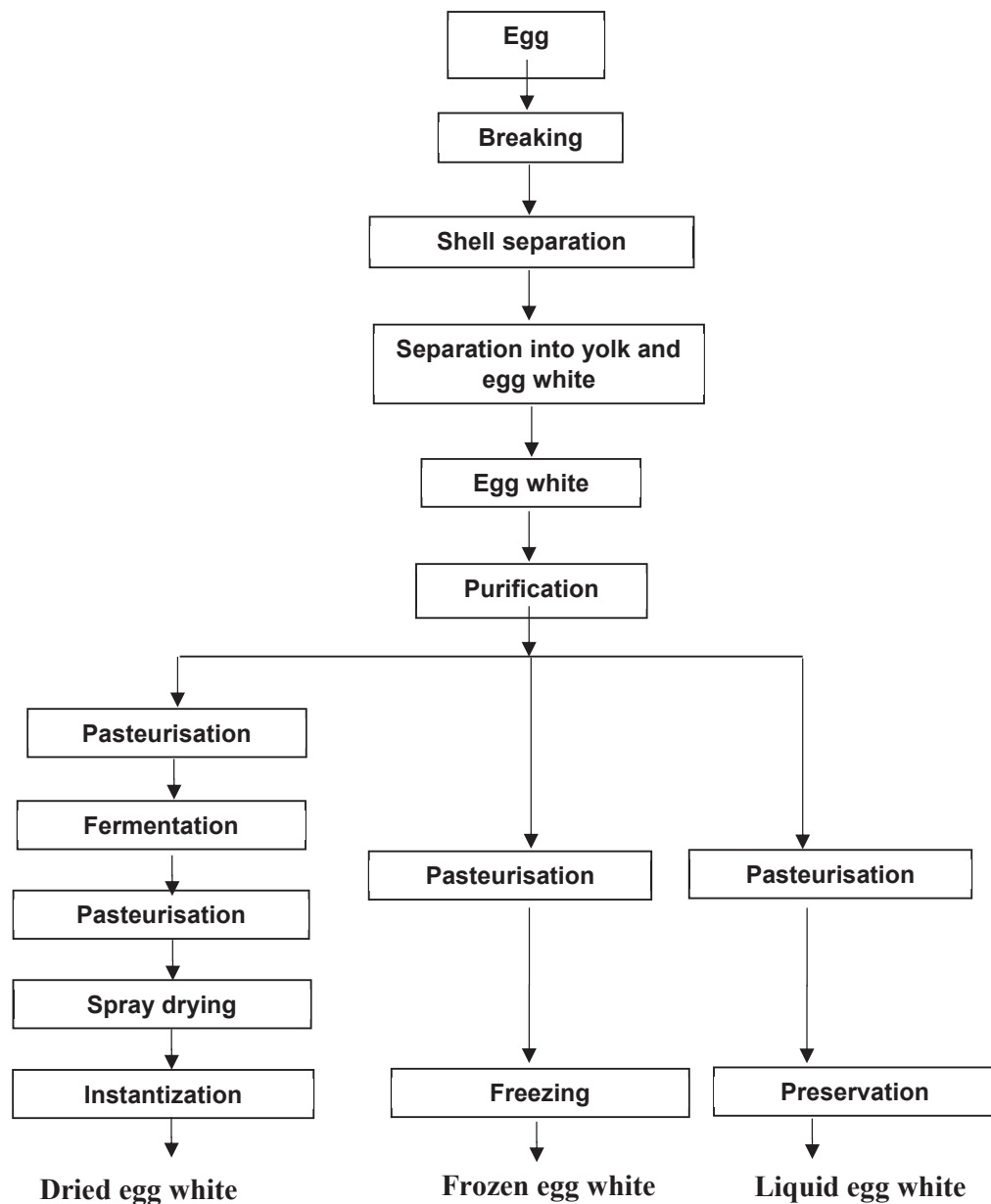


Figure 2.4: Schematic flowchart of production of commercial egg white products

2.9.2 Frozen and liquid egg white

As shown in Figure 2.4, the egg white after purification is pasteurised at around 57°C for 3-5 minutes or 63°C for 1 minute to reduce microbial count, then it is frozen fast at -40°C. Frozen egg white's shelf life is up to a year at -15 to -18°C storage temperature. (Stadelmann & Cotterill, 1977). There is no significant thickening of egg white after

thawing (Belitz et al., 2009). Liquid egg white is pasteurised as described above, normal pasteurisation does not negatively affect the functional properties of egg white proteins.

2.10 Conclusion of Literature review

Egg white proteins are regarded as one of the highest quality source of protein in the human diet because they provide a balanced amount of essential amino acids that are readily digestible and adsorbed by the human body. There are about 40 different types of proteins found in egg white liquid with its total protein content of about 10%. Apart from the high nutritional values, egg white proteins possess multiple functional properties, such as binding, adhesion, gelation, foaming and emulsification. Therefore, they are widely used as one of the key ingredient in the food industry. However, the emulsifying property of EWP is considered poor when compared to other proteins (e.g. milk proteins). Although several studies have been carried out to improve the emulsifying property of egg white proteins. There are still some knowledge gaps that need to be investigated when egg white is used as an emulsifier. This review provides an overview of some factors affecting the formation and stability of emulsions prepared with egg white proteins.

Chapter 3. Effects of pH and heat treatment on EWL

3.1 Abstract

This study investigated the effect of pH (pH 2 to 11) on the physical and electrical charge properties of egg white liquid (EWL) containing different egg white protein (EWP) concentrations (0.5, 1, 2, 3, 4, 5 and 10% w/w). The pH adjusted EWL solutions were characterised by ζ -potential and turbidity measurements. The results of the study revealed that for all the protein concentrations used, ζ -potential was close to zero at pH 5, which is the isoelectric point of most egg white proteins that results in protein aggregation and precipitation. At pH 2, ζ -potential values of +27.1 - + 33.4 mV were obtained in EWL containing different protein concentrations (0.5 - 10%) with no significant differences. On the other hand, above pH 6, EWP possessed net negative charges with negative ζ -potential being increased as pH increased from 6 to 11 with the highest net charge of around -34.8 mV. The spectral absorbance (turbidity) which was measured at 600 nm revealed that for all the protein concentrations used, highest turbidity was obtained at acidic pH (3, 4 and 5). At alkaline pH (7, 8, 9 and 10) the EWL was transparent. The effect of heat treatment and holding time on the denaturation of EWP was also studied by heating EWL at different temperatures (57-62°C) for heating times (0-19 minutes). Higher turbidity and protein aggregation were observed as temperature increased from 57 to 62°C and the heating time increased from 5 to 19 minutes. At 60°C, EWL began to thicken and after heating for 5 minutes coagulation and gelation occurred rapidly. Partial denaturation of EWP was achieved when the EWL was heated at around 57-58°C for below 5 minutes.

3.2 Introduction

Egg white proteins are recognised as one of the best quality proteins because they contain a balanced amount of essential amino acids (Raikos et al., 2007; Belitz et al., 2009), reasonable amounts of water soluble vitamins (thiamine, niacin, pantothenic acid, riboflavin, folic acid, etc) (Campbell et al., 2003; Belitz et al., 2009) and are rich in minerals (e.g. potassium, sodium, phosphorous, magnesium, manganese, sulphur, chlorine) (Li-Chan & Kim, 2008; Johnson & Ridlen, 2016). Additionally, they possess unique functional properties such as foaming, gelation, emulsification and water binding (Mine, 1997; Strixner & Kulozik, 2011). Therefore, it is an extensively used ingredient in the food industry (Mine, 1995; Cegielska-Radziejewska et al., 2008; Abeyrathne, Lee, & Ahn, 2013).

Like other proteins (e.g. milk proteins), the functional properties of EWP are also affected by some physicochemical factors, such as pH, ionic strength and temperature. The nature and distribution of electrical charges on a protein molecule is greatly influenced by pH. At pH close to the pI of proteins, the protein molecule has a zero-net charge and loses electrostatic repulsive forces, leading to protein aggregation, precipitation and minimal solubility (Croguennec, Nau, & Brulé, 2002; Pelegrine & Gasparetto, 2004; Machado et al., 2007). EWP is a mixture of different proteins, thus having varying pI. Some have pI around pH 4.1-4.5 (ovalbumin, ovomucoid, ovomucin) while others have at around pH 6 (ovotransferrin) and at pH 10 (avidin and lysozyme).

Eggs are subjected to heat treatment in the food industry to obtain specific desirable organoleptic attributes or to ascertain microbial safety (usually by pasteurization) (Van der Plancken, Van Loey, & Hendricks, 2006; Lechevalier et al., 2017). However, liquid egg white pasteurization is problematic owing to its sensitivity and instability at effective pasteurization temperature range (Stadelman and Cotterill, 1995). As a result, pasteurization usually occurs between 53 and 55°C for 2-10 minutes to maintain a 5 to 6 decimal reduction of vegetative microorganisms (e.g. *Listeria monocytogenes* and *Salmonella enteritidis*) (Baron et al., 2010). Denaturation and functionality impairment of egg white is a function of temperature and time as desired functional property is lost at temperature as low as 50°C and holding time of 30 minutes or longer; with the loss becoming more rapid at 58.9°C (Mine et al., 1990; Lechevalier et al., 2017a). At around 60°C, EWL begins to thicken, coagulates rapidly and subsequently forms gels with distinctive textures (Palumbo, Beers, Bhaduri & Palumbo, 1996; Abbasnezhad, Hamdami, Monteau, & Vatankhah, 2015). Denaturation by heat causes the protein secondary and tertiary structures to partially or completely unfold to expose the reactive amino acid groups (e.g. sulfhydryl groups and hydrophobic amino acids) from their interior, leading to disulphide and hydrophobic interactions between the denatured protein molecules (Chang et al., 2016). The disulphide bonds initiate thermal aggregation of EWP, while electrostatic and hydrophobic interactions govern the formation gel network after protein denaturation (Van der Plancken et al., 2006).

Although studies on the effects of pH and heat treatment on EWP have been well documented, there is still a gap of knowledge on the effect of pH on physical changes and electrical properties of EWL containing different protein concentrations. Secondly,

investigations on the effect of temperature and heating time on degree of denaturation of EWP still needs further studies. Therefore, the objective of this study was to investigate the effect of pH on protein aggregation and precipitation in EWL containing different EWP concentrations (0.5, 1, 2, 3, 4, 5 and 10% w/w). Secondly, the effect of heat treatment on partial protein denaturation of EWL was investigated to understand the impact of temperature and heating time on the degree of EWP denaturation.

3.3 Materials and Methods

3.3.1 Materials

Egg white liquid (EWL) which was pasteurized and contained 10% w/w protein was purchased from Zeagold Foods (New Zealand). Analytical grade reagents such as hydrochloric acid (HCl) and sodium hydroxide (NaOH) were purchased from Thermo Fisher Scientific Inc. (Auckland, New Zealand).

3.3.2 Sample preparation

Original EWL containing 10% w/w protein was diluted with distilled water to different protein concentrations (0.5, 1, 2, 3, 4 and 5% w/w). The original EWL was also used as control without any dilution. The effect of pH on protein aggregation in EWL was examined by adjusting the original and diluted EWL solutions at pH 9.0 ± 0.02 to pH 2, 3, 4, 5, 6, 7 and 8 using HCl and to pH 10 and 11 using NaOH. A pH meter (Sartorius Basic pH meter pH-20) was used to measure the pH of all the EWL solutions. All samples prepared were analysed for zeta potential and turbidity measurements after the pH adjustment.

3.3.3 Zeta potential (ζ -potential) measurements

The ζ -potential (zeta potential) of the pH adjusted EWP solutions described above was analysed for their electrical net charge by measuring electrophoretic mobility using a Zetasizer Nano ZS90 (Malvern Instruments Ltd., Worcestershire, UK). EWP solutions containing 0.5% and 1% protein concentration were diluted at a ratio of 1:10 and the rest of the EWP solutions at a ratio of 1:100 (v/v) before measurements with distilled water of appropriate pH to prevent multiple light scattering effects. The samples were equilibrated inside the instrument for 60 seconds at 25°C before analysis. Triplicate measurements were carried out for all samples .

3.3.4 Turbidity measurement

The pH adjusted EWL solutions with different protein concentrations were analysed to ascertain their optical properties by measuring turbidity. Turbidity was measured using a UV/Vis spectrophotometer (UV-1800, Shimadzu, Japan) at an absorbance of 600 nm. All measurements were carried in triplicates.

3.4.5 Effect of heat treatment on EWL denaturation

The effect of heat treatment on the denaturation of EWP was examined using original EWL containing 10% w/w protein. Firstly, 8 ml of EWL in glass test tubes with a screw cap were equilibrated in a water bath at $20 \pm 1^\circ\text{C}$ for 30 minutes. This was followed by heat-treatment at 30, 40, 50, 53, 55, 57, 59 and 60°C and the times it took to reach the required selective temperature was recorded. The temperature was monitored by inserting a thermocouple thermometer into a test tube containing EWL. When the EWL reached the desired temperature, they were removed and placed immediately in an ice water bath to cool down.

To estimate the effect of heat treatment temperature and holding time on the denaturation of EWP in EWL. EWL was heat treated at different temperatures (55, 57, 58, 59, 60 and 62°C) for different times (0 -19 minutes) as shown in Table 3.1. In this experiment, 10 ml of EWL in glass tubes were incubated at desired temperatures ($\pm 1^\circ\text{C}$) in a water bath with a thermocouple thermometer inserted to monitor when it reached the desired temperature. At each temperature used, when the EWL reached the required time, the test tubes were taken out immediately and placed in an ice bath to cool down and stop further denaturation. Then the cooled samples were centrifuged at $4000 \times g$ for 20 minutes at room temperature to visually observe the extent of protein aggregation and precipitation formed.

Table 3.1: Heat treatment of egg white liquid (EWL) at different temperatures at different holding times

Temperature ($^\circ\text{C}$)	Time (minutes)
57	0, 1, 2, 3, 4, 6, 7, 8, 9, 10
58	0, 1, 2, 3, 4, 6, 7, 8, 9, 10
59	0, 1, 2, 3, 4, 6, 7, 8, 9, 10
60	0, 1, 3, 5, 7, 9, 11, 13, 15, 17, 19
62	0, 1, 3, 5, 7, 9, 11, 13, 15, 17, 19

3.4.6 Statistical analysis

The results were analyzed using Minitab 17.1.0 statistical software (Minitab, Inc., USA). Statistical analysis was determined by analysis of variance (ANOVA) to determine statistical difference at ($p < 0.05$). Significance between means was determined using the Turkey's HSD test. All experiments were carried out in at least duplicates and all measurements were carried out in triplicates for each duplicate experiment.

3.5 Results and Discussion

3.5.1 Effect of pH on zeta potential of EWL solutions

ζ -potential measures the degree of attraction or repulsion between surface charges of the dispersed particles and those of the dispersion medium. Higher the ζ -potential equates better stability of dispersed particles. Generally, ζ -potential values $\geq +30$ mV or -30 mV are sufficient to ensure an electrostatic stability of dispersed particles (Roland, Piel, Delattre, & Evrard, 2003; Silva, 2012). However, when ζ -potential is low, attraction forces exceeds repulsion causing the dispersion to destabilise and flocculate. On the other hand, higher ζ -potential indicates more stability of the dispersed particles in a suspension (Silva, 2012). In this study, the effect of pH (2, 3, 4, 5, 6, 7, 8, 9, 10 and 11) on ζ -potential of egg white solutions containing different protein concentration (0.5, 1, 2, 3, 4, 5 and 10% w/w) were estimated, and the results obtained are shown in Figure 3.1.

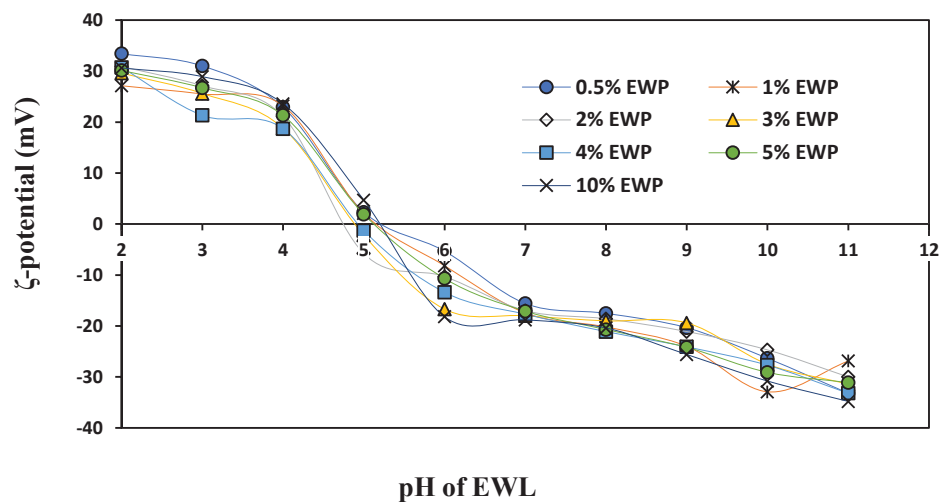


Figure 3.1: ζ -potential of pH adjusted EWL at different protein concentrations (0.5, 1, 2, 3, 4, 5 and 10% w/w). Each data point is mean $\pm$ standard deviation of two independent measurements with triplicates ($n=6$).

The original EWL at pH 9.0 containing 10% w/w protein had a ζ -potential of -25.6 mV, which increased to -30.8 and -34.8 mV as pH increased from 10.0 and 11.0, respectively. Most food protein molecules have a negative net charge at alkaline pH (Fennema, 1993).

On the other hand, when the pH was reduced, zeta potentials of EWL solutions were +30.6, +28.9, +23.6 and +4.7 mV at pH 2, 3, 4 and 5, respectively. At pH 5, ζ -potential (4.7 mV) was close to zero charge which is the pI of most egg white proteins. A similar trend was reported in the ζ -potential results obtained in EWL solutions containing 0.5, 1, 2, 3, 4, 5% w/w protein concentration with highest positive ζ -potential obtained at pH 2 and highest negative ζ -potential obtained at pH 11. Highest positive ζ -potential values of +33.4, +27.1, +30.9, +29.6, +30.7 and +30.1 mV were obtained at pH 2 in EWP solutions containing 0.5%, 1%, 2%, 3%, 4% and 5% protein, respectively. On the other hand, highest -ve ζ -potential values of -33.2, -26.9, -30.0, -31.4, -33.2 and -31.1 mV were obtained at pH 11 in egg white protein solutions containing 0.5%, 1%, 2%, 3%, 4% and 5% protein, respectively. The findings were in agreement with the results of Altalhi (2013) who investigated the effect of pH on EWL solutions containing 10% w/v protein concentration and reported at above pH 5, ζ -potential became more negatively charged as pH increased while it became more positively charged at below pH 5 as pH decreased. ζ -potential value close to zero at pH 5 was also reported. Generally, proteins have either an overall net positive or net negative charges at low or high pH, resulting in strong electrostatic repulsive force that help in keeping the proteins molecules apart, thus improving protein solubility (Damodaran, 2005; Yuliana et al., 2014). However, at pH close to the pI, aggregation of proteins occurs as a result of strong intermolecular interactions, leading to less protein solubility and stability (Damodaran, 2005). In summary, EWP protein concentration had no significant influence on the ζ -potential of EWP but ζ -potential was greatly influenced by pH changes.

3.5.2 Turbidity of EWL solutions

Turbidity assay is an important approach in identifying indirectly the aggregation of proteins during food processing. It is described as haziness or cloudiness of fluids produced by individual particles (dissolved solids or totally suspended) which can be inversely proportional to the transmittance of the protein solution (Akkouche, Madani & Aissat, 2012; Wu, Zhao, Yang, Yan & Sun, 2015). An adjustment of the pH of egg white is carried out to promote the isoelectric precipitation of specific proteins, produce desired functional property and intensify some processing techniques such as enzymatic operations (Cotterill,

Gardner, Cunningham & Funk, 1959). Variation in pH of EWP is responsible for the change its physical properties and turbidity is an example of physical changes in egg white. Usually turbidity occurs at pH close to pI (Cotterill et al., 1959). In this study, the effect of pH on EWL solutions containing different protein concentrations (0.5, 1, 2, 3, 4, 5 and 10% w/w) was carried to estimate the extent of protein aggregation and precipitation through turbidity measurement using a spectrophotometer and visual observation. The results for absorbance (turbidity) are shown in Figures 3.2 and 3.3.

The spectral absorbance value measured at 600 nm of the original EWL (10% w/w protein) solution at pH 9.00 was 0.372, while high absorbance values of 2.42, 2.57 and 2.69 were obtained at pH 3, 4 and 5, respectively, indicating protein aggregate formation and high turbidity. As shown in Figure 3.3, original EWL with its initial being ~pH 9.0 was transparent, but was more opaque and turbid at pH 5, followed by pH 4 and then pH 3. Apparently, this is because the pI of most EWPs is around pH 4 and 5 (Mine, 1995, Belitz et al., 2009). At pH closer to the pI, electrostatic repulsive forces become weak as the proteins become less charged causing protein agglutination, aggregation and loss of solubility (Croguennec et al., 2002; Van der Plancken et al., 2006). In the same respect, high turbidity was observed at pH 4 and 5 for 0.5% protein EWL solution; pH 4, 5 and 6 for 1, 2, 3, 4 and 5% protein EWL solution was more turbid at pH 4 and 5; the other EWL solutions containing different protein concentration were found to be turbid at acidic pH 3, 4 and 5, and transparent at pH 7, 8, 9 and 10 just like the 10% protein EWL solution. Secondly, turbidity across all pH increased as protein concentration increased, because more proteins were available for aggregation in concentrated solutions than in lesser (dilute) EWL protein concentration. In general, highest absorbance and turbidity was observed at pH 5. The results corresponded with those reported by of Bovskova and Mikova (2011) and Altalhi (2013) indicating that high turbidity was observed at acidic pH 3, 4 and 5. The results of this investigation, indicated that regardless of the protein concentration of EWL, highest turbidity was observed at acidic pH. Additionally, EWL solutions containing more protein concentrations were more turbid than those containing lesser protein concentration. At alkaline pH 7 to 10, absorbance values among the different protein solutions were similar.

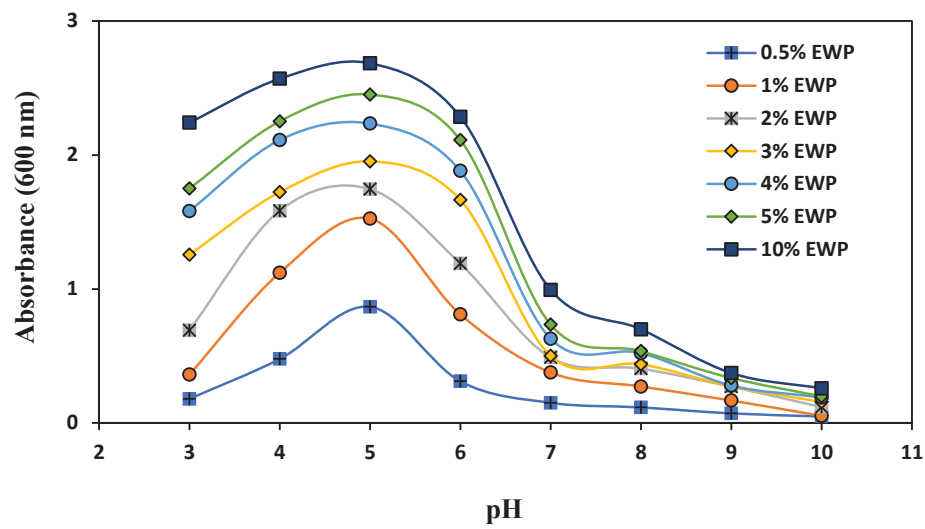


Figure 3.2: Turbidity of EWL containing different EWP concentrations (0.5, 1, 2, 3, 4, 5 and 10%) at different pH ranging from pH 3 to 10. Each data point is mean $\pm$ SD for n=6.

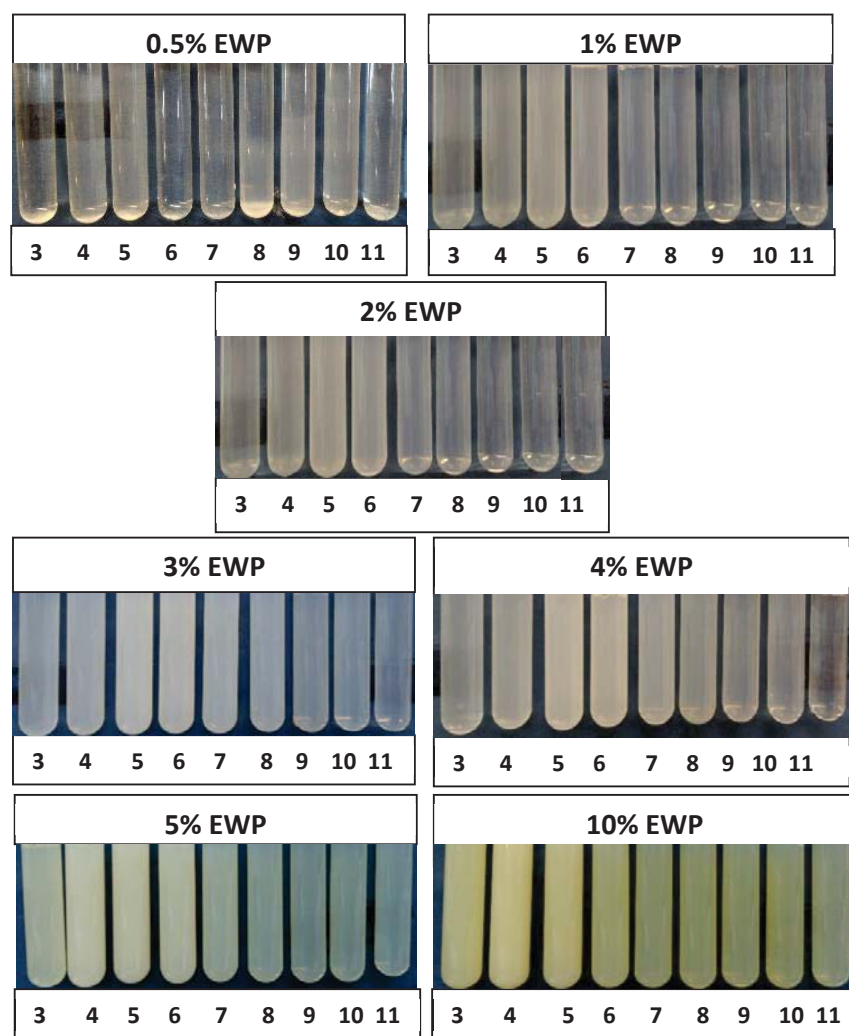


Figure 3.3: Photographs of EWL solutions containing different EWP concentrations at pH levels ranging from pH 3 to 11.

3.6 Effect of heat treatment on EWL

The pasteurization process of egg white products is a combination of temperature and holding time. The process is difficult due to the limited heat tolerance of egg whites and the need to destroy pathogenic microorganisms especially the *Samonella spp.* (Cunningham, 1995; Belitz et al., 2009). Commercially, egg white is pasteurized at temperatures ranging from 52 to 56 for 3 to 5 minutes. At above this temperature and time, denaturation and gelation egg white protein occur exceedingly. To investigate the extent of protein denaturation in EWL in relation to temperature and holding time, firstly, the time taken for 8 ml of EWL (10% w/v protein) equilibrated to $20 \pm 1^\circ\text{C}$ for 30 minutes in a water

bath to reach desired temperatures (30, 40, 50, 53, 55, 57, 59 and 60°C) was determined. The temperatures used in this study were chosen because they were the pasteurization temperatures used commercially for egg white products. As shown in Figure 3.4, it took 71 seconds for EWL to get to 50°C from 20°C, 98 seconds for 55°C and 190 seconds to get to 60°C. The times corresponded to the pasteurization time of 2 to 3 mins.

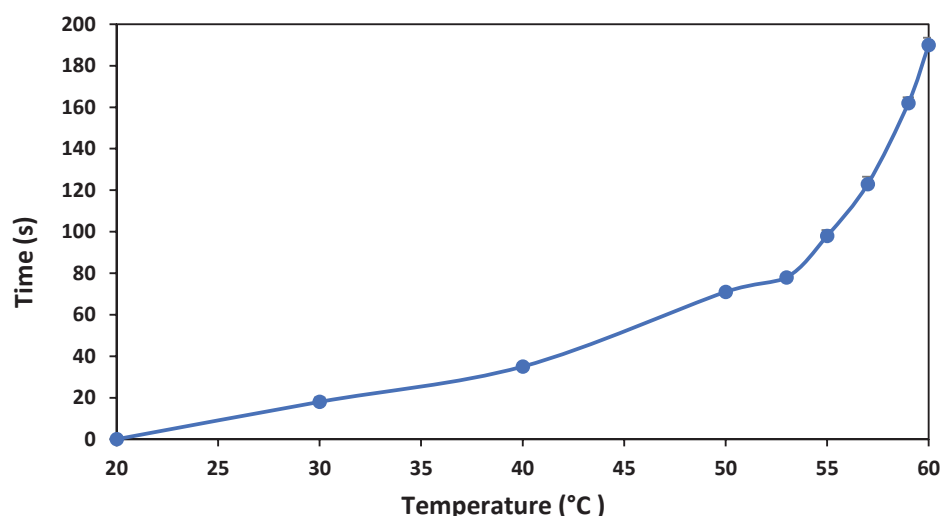


Figure 3.4: Time taken for EWL solution at 20°C to reach desired temperatures (57, 58, 59, 60 and 62°C). Each data points represents mean $\pm$ SD (n=3).

The effect heat treatment on EWL was further studied to determine the holding time egg white proteins denature. For this, EWL solutions (10% w/w) at 20°C was heat treated at varying temperatures 57, 58, 59, 60 and 62°C at holding time ranging from 0-19 minutes, the results are shown in Figure 3.5. At 57 and 58°C, very little or no soluble protein aggregates was seen settling at the bottom of the test tube at holding time 0 - 10 minutes. At 59°C, soluble protein aggregates could be seen at the test tube bottom from 8 minutes and at 10 minutes more soluble aggregates and coagulation was seen. When temperature increased to 60°C, considerable denaturation and coagulation of proteins occurred rapidly from 5 minutes, and increased as holding time increased. After 17 minutes EWL gelled. To further observe extent of coagulation of EWL, the heating temperature was extended to 62°C. At 62°C, EWL became completely opaque and gelled at all heating times. This observation was in agreed with previous studies reported by Croguennec et al. (2002) and

Kaewmanee et al. (2011). They reported increase in protein aggregation with increasing time. Thermal aggregation of EWP occurs by the formation of intermolecular β -sheet protein structure governed by disulphide bonds, hydrophobic interactions and ionic interactions (Van der Plancken, Van Loey, & Hendrickx, 2005). The findings of this study confirm temperature and time to be important in EWL heat treatment. EWP solution was heat treated successfully without aggregation or coagulation at 57 and 58°C below 10 minutes holding time, at 60°C EWL could be heated successfully at 3 minutes with minimal denaturation. At these successful temperatures, EWP can still maintain its useful functional property.

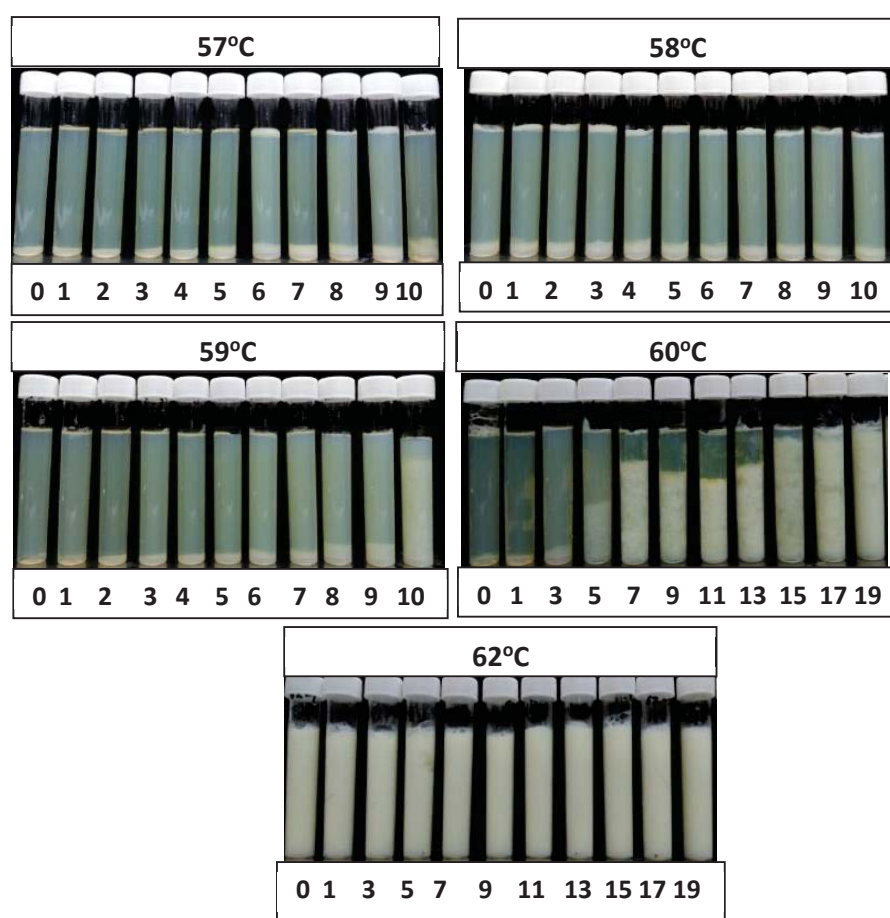


Figure 3.5: Photographs of time dependent (0-19 minutes) changes in turbidity and denaturation of EWL (10% w/w protein) due to heat treatment (57, 58, 59, 60 and 62°C).

3.7 Conclusions

The physicochemical properties of EWP are significantly affected by pH and heat treatment. The results revealed ζ -potential was significantly affected by pH, regardless of the protein concentrations used. At pH 5 which is close to the pI of most egg white proteins, ζ -potential values were close to zero; above it the values were negative and below pH 5 they became positive. Reducing protein concentration of EWL did not have significant effect on ζ -potential.

Absorbance of EWL measured at 600 nm showed that the highest turbidity was obtained at acidic pH 3, 4 and 5, with the highest at pH 5, followed by pH 4 for all EWL solutions containing different protein concentrations. Turbidity of EWL increased significantly as protein concentration increased. At alkaline pH, absorbance values of the different protein solutions were similar, but significant difference could be seen at acidic pH 3, 4 and 5 among different protein concentrations.

The impact of heat treatment and holding time on protein denaturation of EWL (containing 10% w/w protein) at different temperatures was also studied. At 57 and 58°C, EWL were stable against protein aggregation even at 10 minutes holding time. It was also observed that EWL solution can be successfully heat treated at 60°C for 3 minutes but above this temperature and time rapid coagulation and then gelation of proteins occurred which can result in loss of functional property.

Overall, the results of this study provided meaningful information on the application of temperature and time for the partial denaturation of EWP. Also, the results revealed high zeta potential at acidic pH 3, 4 and 5 and alkaline pH 9 and 10. This information can be applied in producing more desirable, innovative EWL products with maximum stability against aggregation.

Chapter 4. Reduction of visible aggregates formed during emulsification using various methods

4.1 Abstract

Various methods (pre-homogenisation heat treatment and variation in oil and protein concentrations) for reducing the formation of aggregates during emulsification using EWP as emulsifiers were investigated. Emulsions produced were analysed for their visual appearance, droplet size, ζ -potential and microstructure. Pre-heating of EWP prior homogenisation had no significant effect on formation of aggregates and droplet size of emulsions stabilised by EWP. However, oil concentration had a significant effect on the formation of aggregates and droplet size ($\sim 0.32 \mu\text{m}$). Smaller droplet size and less aggregates were produced at 5% oil concentration. Emulsions containing higher oil concentrations were susceptible to formation of large visible aggregates and droplet size. This was attributed to coalescence and flocculation possibly by bridging caused by insufficient proteins to form dense protective layer around the droplets. The effect of protein concentration (1 and 3% w/w) at different oil concentrations (1, 3, 5, 6, 7 and 10% w/w) was also investigated. Emulsions prepared with 1% EWP resulted in smaller droplet and aggregates at oil concentration below 7%. Increasing oil and protein concentrations significantly ($p < 0.05$) increased droplet size and aggregates. Phase separation was observed 1 hour after preparation in emulsions stabilised by 3% EWP and prepared at 3, 5, 6, 7 and 10% w/w oil concentrations except at 1% oil. In contrast, emulsions stabilised by 1% EWP were stable against phase separation at all different oil concentrations used (1, 3, 5, 6, 7 and 10% w/w) even after 1 week.

4.2 Introduction

The proteins of egg white are natural and major sources of high quality, digestible proteins and essential nutrients, containing about 9.7-10% protein in weight in EWL (Mine, 1995). Besides their nutritional benefits, they possess multiple unique functional properties, such as foaming, gelling and emulsifying properties. Thus, they are used as key ingredients in baked foods, meat products, meringues, salad dressings and ice cream (Drakos & Kiosseoglou, 2006). Egg white is a colloidal mixture of about 40 different proteins. Three major proteins contributing to its functionality are ovalbumin (54%), ovotransferrin (12%) and lysozyme (3.5%). Ovalbumin is the major protein responsible for its unique functionality. This phosphoglycoprotein consists of 385 amino acid residues with a

molecular weight of about 45 kDa, one disulphide bond and four SH groups buried deep within its structure (Li-Chan, Powrie, & Nakai, 1995; Belitz et al., 2009). Egg whites are commercially available in liquid, frozen or dried state (Stadelman & Cotterill, 1995; Lechevalier et al., 2017a).

Although, EWP boasts of excellent gelation and foaming properties, its emulsifying properties are considered poor when compared to other proteins, such as whey, casein or soy (Chang et al., 2016). Several researchers have investigated various methods for improving the emulsifying properties of EWP, such as heat treatment (Kato et al., 1989; Mine, 1997; Chang et al., 2016; Lechevalier et al., 2017a); high intensity ultrasound (Arzeni, Perez, & Pilosof, 2012); pH treatments (Mine et al., 1991; Mine, 1997; Chang et al., 2016); Maillard reactions (Kato, Minaki, & Kobayashi, 1993; Nakamura & Kato, 2000; Al-Hakkak & Al-Hakkak, 2010; O'Charoen, Hayakawa, & Ogawa, 2015; Padala et al., 2009; Niu et al., 2015; Niu et al., 2016); and enzymatic hydrolysis (Chen et al., 2012; Chang et al., 2017). Nevertheless, up until now there are no information and supporting data available on formation and reduction of droplet aggregates formed during emulsion preparation using EWP as emulsifiers. Hence, this study investigates various methods of reducing the formation of aggregates produced during preparation of EWP emulsion.

Generally, environmental conditions, such as heat treatment, pH, protein concentration, oil concentration and protein to oil ratio, are known to have profound effect on emulsifying properties of proteins (Qian & McClements, 2011; Ettoumi, Chibane & Romero, 2016). In food processing, heat treatments are not only applied for pasteurisation but also for its effects on structural changes and functional properties of proteins (Dickson, 1994; Van der Placken et al., 2007). In order to understand the relationship between heat treatment and protein functionality, several studies have investigated the effect of dry heating on the structural, foaming and gelling properties of EWP (Kato et al., 1989; Hammershoj et al., 2006; Van der Placken et al., 2007; Desfougeres et al., 2008; Baron et al., 2013). Increased foaming ability and stability were reported at heating temperature greater than 70°C (Kato et al., 1989; Desfougeres et al., 2008). Furthermore, protein aggregation and increase in surface hydrophobicity and flexibility were also reported (Hammershoj et al., 2006; Van der Placken et al., 2007). However, fewer studies have focused on the effect of heat treatment on emulsifying properties of EWP, especially with respect to formation of aggregates during emulsion preparation. Recent study by Lechevalier et al. (2017a)

investigated the effect of dry heating (at 60, 70, 80 and 90°C for 1, 2, 5 and 10 days). They reported smaller droplet size and increased creaming stability at 70°C and higher as heating duration increased. The increased emulsifying ability was attributed to increased surface activity, hydrophobicity and flexibility of the heated EWP.

Proteins are key ingredients used in emulsion preparation as they can act as both emulsifying and stabilising agents (Dickson, 1992; Tadros, 2005). Some proteins are amphiphilic surface-active molecules that can diffuse onto the oil-in-water (O/W) interface to reduce the interfacial tension and form a thick protective layer around the oil droplets preventing oil droplet coalescence and flocculation (McClements, 2005; Tadros, 2013), thus ensuring long term stability of the prepared emulsions. Many researchers have studied the emulsifying properties of proteins from different sources, such as milk (whey and casein), plants (potato, soy, peas), meat and eggs (egg white) (Mine, 1991; Guo & Mu, 2011; Amine, Dreher, Helgason, & Tadros, 2014). Several researchers have established the fact that the concentration of an emulsifier determines the droplet size distribution, interfacial properties, rheology and creaming stability of emulsions (Dickson & Golding, 1997; Sun & Gunasekaran, 2009; Guo & Mu, 2011; McClements, 2015). Bridging flocculation and coalescence of emulsions have been attributed to lack of sufficient emulsifier in the continuous phase. On the other hand, depletion flocculation could arise when there are excess non-adsorbing emulsifiers in the continuous phase. Hence, larger emulsion droplets and instability can also occur in the presence of sufficient emulsifiers (Sun & Gunasekaran, 2009; Dickson, 2009; Dickson, 2010). However, to the best of the author's knowledge, there are no sufficient scientific and technical information available on the effect of EWP concentration with regards to formation or reduction of aggregates formed in EWP-stabilised emulsions.

Another important ingredient in O/W emulsions that affects the appearance, flavour, texture and stability is oil. A previous study reported by Mine et al. (1991) demonstrated increased emulsifying ability and stability with increasing oil concentration, especially with high protein concentration when emulsions were prepared with various EWP concentrations (0.1, 0.5, 1, 2, 3, 4 and 5% w/v) and oil concentrations (12.5, 25 and 50% w/w). Romero et al. (2017) also reported smaller droplet size and higher stability with increasing oil concentration in emulsions prepared with 30g/kg and different oil concentrations (450, 550,

650 and 750g/kg) at pH 3. Nevertheless, no investigation has reported the effect of oil concentration on formation or reduction of aggregates formed during emulsification.

Thus, the aim of this present study was to investigate the effects of pre-homogenisation heat treatment, oil concentration and protein concentration on the formation and reduction of aggregates formed during preparation of EWP emulsions. The information obtained will provide an understanding on how to control and minimise the formation of aggregates such that there is a high potential of practical application of EWP as emulsifiers in food products.

4.3 Materials and Methods

4.3.1 Materials

Pasteurised EWL containing 10% (w/w) protein (Zeagold Foods, New Zealand) was purchased from a local supermarket. Canola oil was also purchased from a local supermarket and used as oil phase.

4.3.2 Effects of pre-homogenisation, heat treatment and oil concentration

The aim of this present experiment was to investigate the effects of both pre-heating EWP solution prior homogenisation and oil concentration on the emulsifying properties of EWP in relation to reduction of droplet aggregates. EWL was diluted with distilled water to prepare EWP solution containing 1% w/w protein. The EWP solution prepared was heat treated at 60°C for 30 minutes (prior homogenisation) in order to induce partial denaturation which may increase EWP's flexibility, surface hydrophobicity and emulsifying properties. Thus, to produce emulsions with less aggregates and good emulsifying properties of EWP, heating temperature was fixed for 60°C which is around the pasteurisation temperature used in the egg industry (Cunningham, 1995; Baron et al., 2010). The protein concentration (1% w/w) was chosen to limit extensive protein coagulation and gelation as studies have reported rapid coagulation of EWP at high temperature and high protein concentrations (Belitz et al., 2009). Additionally, the effect of various amounts of oil concentration (5, 10, 15 and 20% w/w) on EWP's emulsifying properties and reduction of aggregates was also studied.

4.3.2.1 Preparation of emulsions

EWL (10% w/w protein) was diluted with distilled water to a protein concentration of 1% (w/w). The protein solution was heated to 60°C for 30 minutes in a water bath followed by

cooling immediately in an ice bath. Different oil phase emulsions were prepared by mixing canola oil with egg protein solution at a ratio of 5:95, 10:90, 15:85 and 20:80. The mixtures of oil and protein solutions were pre-homogenised for 2 minutes to form coarse emulsions using an Ultra-Turrax blender (IKA T25 Basic, Staufen, Germany) (Figure 4.1a) at 13,000 rpm. The coarse emulsions obtained were immediately homogenised 4 times using a two-stage high pressure homogeniser (APV-2000, APV Manufacturing, Poland) (Figure 4.1b) at 500/50 MPa (first/second stages) to produce fine emulsions. Control emulsions were also prepared with un-heated EWP solution at various oil concentrations same as the preheat-treated protein emulsions. The prepared emulsions were stored at 4°C until further analysis. All emulsions were prepared in duplicates. The formulations used to prepare the un-heated EWP (UEWP) emulsions and heat-treated EWP (HEWP) emulsions are shown in Table 4.1.

Table 4.1: Formulations of O/W UEWP emulsions and HEWP emulsions prepared with 1% w/w EWP and 4 different oil concentrations (5, 10, 15 and 20% w/w)

Oil to aqueous phase ratio	Canola oil (g)	1% (w/w) protein EWL (g)	Total (g)
5:95	20	380	400
10:90	40	360	400
15:85	60	340	400
20:80	80	320	400



Figure 4.1: (a) Ultra Turrax (IKA T25 Basic, Staufen, Germany) and (b) 2-stage high pressure homogeniser (APV-2000 APV Manufacturing, Poland).

4.3.2.2 Analysis of emulsion droplet size

The particle size of emulsions was measured by laser light scattering technique using a Malvern Mastersizer 2000 (Malvern Instruments Ltd., UK). About 1-5 ml of emulsion samples were injected into a sample dispersion chamber equipped with a flow pump connected to an optical chamber for the measurement of emulsion droplet size. Distilled water was used as the dispersant and 1.465 was used as the refractive index of canola oil. The droplet size was reported as Sauter mean diameter ($D_{3,2}$), span and volume size distribution. The emulsion particle sizes were reported as the average and standard deviation of at least 5 readings made on two independent samples.

4.3.2.3 Microscopic examination of emulsions

The microstructure of both type of emulsions was examined by confocal laser scanning microscopy (CLSM). A small amount of emulsion was placed in a cavity slide and 50 μ L each of 0.2g/L Nile red and fast green staining solutions were added before being covered with a coverslip. Leica DM6000B SP5 confocal laser scanning microscope system running LAS AF software (version 2.7.3.9723; Leica Microsystems CMS GmbH Germany) was used to capture the confocal images of samples with a 100x oil (N.A. 1.40) lens. Nile red and fast green were sequentially imaged through excitation at 488 nm (argon laser) and 633 nm (HeNe 633 laser), respectively, and emission collection at 498-569 nm and 643-787 nm, respectively. ImageJ software version 1.47 with Bio-formats importer plugin was used to analyze the images.

4.3.3 Effects of oil concentration and protein concentrations

To minimise the influence when the protein/oil ratio during homogenisation is low and reduce the formation of visible insoluble droplet aggregates/flocs and large droplet sizes that may occur when the EWP emulsifier is shared among clustered oil droplets, two different EWP concentrations (1 and 3% w/w) and a lower range of oil concentrations (1, 3, 5, 6, 7 and 10% w/w) were considered based on some findings from Section 4.3.2 in order to determine what concentration of oil and/or protein would cause the formation aggregates during homogenisation.

4.3.3.1 Preparation of emulsions

For this present study, two sets of emulsions stabilised by EWP were prepared to investigate the concentration of oil and/or protein concentration that causes the formation of droplet aggregates during preparation of emulsions. For the first set of emulsions, original EWL

(10% w/w protein) was diluted with distilled water to a protein concentration of 1% (w/w). Then O/W emulsions were prepared by mixing canola oil into each protein solution at an oil to protein ratio of 1:99, 3:99, 5:95, 6:94, 7:93 and 10:90, then the protein solution/canola oil mixture was homogenised to obtain fine emulsions as described in Section 4.3.2.1. For the second set of emulsions, original EWL (10% (w/w) was diluted to 3% (w/w) protein concentration. The protein solution was mixed with canola oil at the same oil/protein ratios used in the first set of emulsions and then homogenised to obtain fine emulsions. The formulations of emulsions used are shown in Tables 4.2 and 4.3. All emulsions were prepared in duplicate and stored at 4°C until analysis.

Table 4.2: Formulations of O/W emulsions prepared with 1% (w/w) EWP and various oil concentrations (1, 3, 5, 6, 7 and 10% w/w)

Oil to aqueous phase ratio	Oil (g)	EWL (g)	Water (g)	Total (g)
1:99	4	39.6	356.4	400
3:97	12	38.8	349.2	400
5:95	20	38.0	342.0	400
6:94	24	37.6	338.4	400
7:93	28	37.2	334.8	400
10:90	40	36.0	324.0	400

Table 4.3: Formulations of O/W emulsions prepared with 3% (w/w) EWP and various oil concentrations (1, 3, 5, 6, 7 and 10% w/w)

Oil to aqueous phase ratio	Oil (g)	EWL (g)	Water (g)	Total (g)
1:99	4	118.8	277.2	400
3:97	12	116.4	271.6	400
5:95	20	114.0	266.0	400
6:94	24	112.8	263.2	400
7:93	28	111.6	260.4	400
10:90	40	108.0	252.0	400

4.3.3.2 Particle size and ζ -potential measurements

The particle size measurements of emulsions were carried out the following day after emulsion preparation by laser light scattering technique using a Malvern Mastersizer 2000 (Malvern Instruments Ltd., UK) as previously described in Section 4.3.2.2. The Sauter mean diameter ($D_{3,2}$), volume weight diameter ($D_{4,3}$) and particle size distribution were used to evaluate the emulsion droplet size. The results of emulsion droplet size were reported as the average and standard deviation of at least 3 readings made on two independent samples. The electrical charge (ζ -potential) of the emulsions was measured using a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd, Worcestershire, U.K) as previously described in Section 3.2.2.1. Prior to measurement, the emulsion samples were diluted with distilled water to a ratio of 1:100. All measurements were carried out in at least duplicate on two independent samples.

4.3.3.3 Creaming stability

To investigate the creaming stability, 10 ml of each prepared fresh emulsion was placed into a cylindrical plastic test tube and then sealed to prevent evaporation with a plastic cap. The emulsions samples were stored for a period of 2 weeks at room temperature (20°C). The extent of phase separation and creaming were determined by visual observations.

4.3.3.4 Statistical analysis

All experiments were carried out in at least duplicate. Samples were measured in duplicate from freshly prepared emulsions. All data were presented as mean $\pm$ standard deviation and analysed statistically by analysis of variance (ANOVA) using Minitab statistical software version 17 (Minitab Inc., USA). Significant difference between means were separated by Turkey's HSD method at a confidence level of 95% ($p < 0.05$).

4.4 Results and Discussion

4.4.1 Effects of pre-homogenisation, heat treatment and oil concentration on formation of EWP aggregates

4.4.1.1 Visual observations

Heat treatment has been discovered to be an important step in increasing the surface activity, surface hydrophobicity and flexibility of globular proteins like egg white proteins (especially ovalbumin and lysozyme), which has been shown to have impact on

aggregation, surface properties and viscosity of the protein (Doi, Tani, Murata, Koseki, & Kitabatake, 1993). In this study, EWP solution (1% w/w) was heat treated at 60°C for 30 minutes to induce partial protein denaturation with the purpose of reducing the visible insoluble aggregates usually formed in EWP emulsions. The pre-heated protein solution was used to produce O/W emulsions with various oil concentrations (5, 10, 15 and 20% w/w). Pre-heating the protein solution to 60°C prior to homogenisation did not reduce the visible aggregates of EWP-stabilised emulsions as seen in Figure 4.2. No noticeable difference in the amount of visible insoluble aggregates could be observed in both the UEWP emulsions and HEWP emulsions, indicating pre-heating of EWP (1% w/w) at 60°C for 30 minutes prior homogenisation was not able to reduce the formation of visible emulsion aggregates.

However, the amount of visible aggregates was observed to decrease greatly as oil concentration was reduced from 20% to 5% in both type of emulsions. Emulsions prepared with 5% oil had very little visible aggregates, while highest aggregates were observed at 20% oil. The reason for the little aggregates formed at 5% oil concentration could be that, at this protein/oil ratio, there was sufficient proteins to completely cover the newly formed oil droplets. Nevertheless, at above 5% oil concentration, there was a low emulsifier/oil ratio meaning the EWP available was insufficient to completely cover the large number of newly formed oil droplets. As a result, the unadsorbed oil droplets could collide together (coalescence) or the adsorbed protein molecules are shared among neighbouring oil droplets (bridging flocculation). Bridging flocculation and coalescence of oil droplets can occur in emulsion systems when the amount of emulsifier needed to completely cover the newly formed droplets is low (Dickson, 2009, 2010). According to Dickson & Golding (1997), there is a critical protein/oil ratio required for full coverage of emulsion droplet surface without any noticeable sign of coalescence or flocculation. In this regard, the critical protein/oil ratio required to fully cover the surface of the newly formed oil droplets without any sign of flocculation or coalescence is 1% (w/w) EWP/5% oil (w/w). Above 5% oil concentration, the protein concentration was low and needed sufficient proteins to completely adsorb onto the large amount of newly formed droplets, causing the large amount insoluble droplets observed at 10, 15 and 20% oil concentrations.

The results obtained are interesting as no other studies have mentioned the presence of these insoluble droplets aggregates in EWP-stabilised emulsions or investigated the factors

responsible for their presence. In general, the results obtained indicate that oil concentration played an important role in the formation of insoluble oil droplet aggregates in EWP-stabilised emulsions. Additionally, less aggregates can be formed at low oil concentration (5% w/w) in EWP emulsions stabilised by 1% (w/w) protein. On the other hand, heating EWP solution at 60°C for 30 minutes to partially denature the globular EWP in order to improve its flexibility, surface hydrophobicity and surface activity had no effect in the formation or reduction of droplet aggregates formed in emulsions stabilised with EWP.

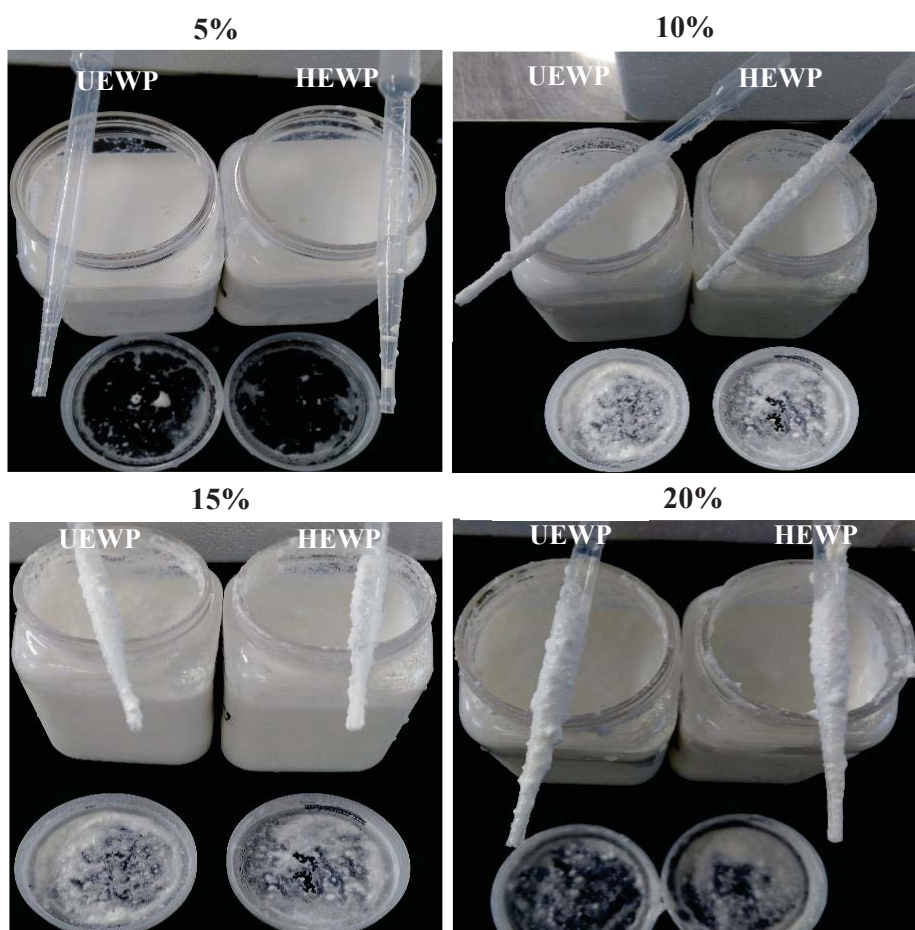


Figure 4.2: Photographs showing insoluble aggregates in UEWP and HEWP emulsions prepared with 1% (w/w) EWP and different oil concentrations (5, 10, 15 and 20% w/w). UEWP and HEWP represents Unheated EWP Heat-treated EWP respectively.

4.4.1.2 Emulsion characteristics

The results of mean particle size ($D_{3,2}$) for the UEWP and HEWP emulsions prepared with various oil concentrations are shown in Table 4.4. Mean particle size increased significantly ($p < 0.05$) as oil concentration increased from 5% to 20% in both un-heated and pre-heated protein emulsions. Particle size of the emulsions ranged from 0.31 to 3.96 μm for UEWP emulsions and from 0.33 to 3.18 μm for HEWP emulsions. Smallest droplet size (0.31 and 0.33 μm) was observed at 5% oil and largest droplet size (3.96 and 3.18 μm) at 20% oil concentration in both UEWP and HEWP emulsions which confirmed the visual observation (Figure 4.2). In Figure 4.2, lesser droplet aggregates were observed at 5% oil and larger visible droplets at 20%. The smaller droplet size at 5% (w/w) oil suggests that EWP concentration was sufficient to cover the emulsion oil droplets and form a sufficient dense layer around it (Dickson, 2010). On the other hand, the large droplets size at 20% (w/w) oil indicates that the EWP concentration was low. As a result, the EWP molecules were not sufficient enough to cover the oil droplets surface and form sufficient adsorption layer. Therefore, the adsorbed protein molecules acted as bridges among the other oil droplets, leading to droplet flocculation (McClements, 2004; Dickson, 2009).

Table 4.4: Mean particle size ($D_{3,2}$) and span values of UEWP and HEWP emulsions prepared with 1% w/w EWP and various oil concentrations (5, 10, 15 and 20% w/w oil)

Emulsion	Oil %	$D_{3,2}$ (μm)	Span
UEWP emulsions	5	0.31 ± 0.01^e	1.92 ± 0.05^c
	10	1.02 ± 0.05^d	2.28 ± 0.26^b
	15	2.57 ± 0.35^c	2.41 ± 0.25^{ab}
	20	3.96 ± 0.67^a	2.44 ± 0.16^{ab}
HEWP emulsions	5	0.33 ± 0.01^e	2.52 ± 0.15^a
	10	0.96 ± 0.04^d	2.40 ± 0.13^{ab}
	15	2.68 ± 0.54^c	2.31 ± 0.11^b
	20	3.18 ± 0.26^b	2.44 ± 0.19^{ab}

Data represents mean $\pm$ standard deviation of two independent measurements ($n=6$). Different superscripts within a column are significantly difference at $p < 0.05$. UEWP: un-heated EWP and HEWP: Heat-treated EWP.

The results obtained collaborated with those obtained in a study by Sun & Gunasekaran (2009), who reported an increase in droplet size with increasing oil concentration in 0.2% (w/w) whey-stabilized emulsions as oil concentration increased from 5 to 40% (v/v). Similar results were also observed in 12% (w/w) gum Arabic-stabilised beverage as oil concentration increased from 20 to 40% (w/w) (Dluzewska, Stobiecka, & Maszewska 2006). Several reasons could be responsible for the observed increase in droplet size with increasing oil concentration: (i) The EWP concentration available was insufficient to cover the surface of the newly formed oil droplets. Hence, the EWP acted as bridges among the oil droplets to cause causing droplet flocculation (McClements, 2004; Dickson 2009); (ii) Increasing oil volume fraction increased the collision frequency of the droplets, leading to enhanced aggregation and flocculation subsequently (Sun & Gunasekaran, 2009); and (iii) lastly, a high oil concentration increases emulsion's viscosity making droplet disruption more difficult (Seekkuarachchi, Tanaka, Kumazawa, 2006; Guo & Muo, 2011).

A comparison of the results for the UEWP and HEWP emulsions indicated that there was no significant difference ($p < 0.05$) in their particle size at 5, 10 and 15% oil concentration. However, significant difference was only observed at 20% oil concentration, in which the un-heated emulsion had a higher particle size (3.96 μm) than those of the pre-heated emulsions (3.18 μm). Several studies have investigated the effect of dry heating on foaming and emulsifying properties egg white proteins (Kato et al., 1989; Hagolle et al., 2000; Talansier et al., 2009; Lechevalier et al., 2017a), but there are no available literatures is on the emulsifying properties of pre-heated egg white protein solution. In a recent study, the effect of dry heating (10 days at 60, 70, 80 and 90°C) on the emulsifying properties of egg white was studied (Lechevalier et al., 2017a). The authors revealed that droplet size decreased significantly with increased dry heating intensity in O/W emulsions prepared with 1.5% EWP and 30% oil. Even at 60°C for 1 day, the droplet size was significantly lower than those of control emulsion without heat treatment. Increased emulsifying ability and stability was also reported in dry heated EWP at 80°C (Kato et al., 1989). The authors attributed these decrease in emulsion droplet size to moderate protein denaturation which increased the surface activity, protein solubility and surface hydrophobicity of egg white proteins. In another study, pasteurisation of whole eggs at 60°C greatly increased the interfacial property leading to increased surface activity and smaller droplet size (Lechevalier et al., 2017b). However, further increase in temperature above 66°C, decreased the surface activity resulting in larger droplet size as the control samples.

Heat treatment of proteins increases the flexibility of the protein molecules causing the proteins to unfold with ease to expose its hidden hydrophobic groups leading to increased surface activity and interfacial activity (Joshi et al., 2012; Tang & Shen, 2013). Heat treatment can also improve the emulsifying properties of proteins by inducing partial hydrolysis, which reduces the protein high molecular weight, increase protein solubility and surface hydrophobicity (Lam & Nickerson, 2013). In this study, there was no impact of pre-heating EWP (60°C for 30 minutes) on the emulsifying property of EWP probably due to the heating time (30 minutes) and low protein concentration (1% w/w) used which was not sufficient to induce the required denaturation needed. Increasing the heating time to at least 1 day may be sufficient to denature and unfold the protein molecules such that the surface activity and interfacial properties are improved.

As shown in Figure 4.3, a monomodal particle size distribution was observed in emulsions prepared with 5% oil. Further increase in oil concentration up to 10%, droplet size curve changed to bimodal distribution with the appearance of a smaller peak (with a range of smaller droplet sizes) and a large peak (range of large droplet sizes). There was a gradual decrease in the volume fraction of the first peak of the bimodal particle size distributions as the oil concentration increased. The range of large droplet sizes in the second peak suggests coalescence of oil droplets or aggregation and flocculation caused by formation protein bridges among oil droplet (Sourdet, Relkin, & Cesar, 2003). The particle size distribution curves collaborated well with the particle size result, as smaller droplet sizes were observed at 5% oil and larger droplet sizes as oil concentration increased further (Table 4.4). Both UEWP and HEWP emulsions showed similar particle size distributions at all the oil concentrations used, confirming the pre-heating of EWP prior to homogenisation did not affect the emulsifying properties (particle size, size distribution and appearance) of EWP emulsions. In both type of emulsions, span values were higher than 1.0 (Table 4.4), indicating high polydispersity and broad droplet size distribution of the samples. The span values ranged from 1.92 to 2.44 for the UEWP emulsion, and 2.52 to 2.44 in the HEWP emulsions as oil concentration increased from 5 to 20%.

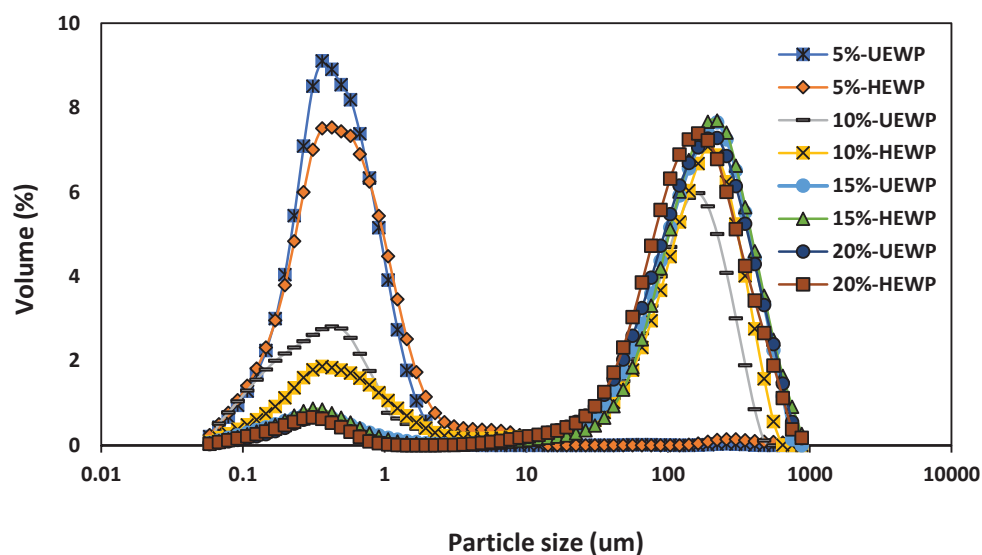


Figure 4.3: Particle size distributions of UEWP and HEWP emulsions prepared with 1% w/w EWP and different oil concentrations (5, 10, 15 and 20% w/w). UEWP and HEWP represents Un-heated EWP and Heat-treated EWP respectively.

To illustrate better the characteristics of the oil droplets in the emulsions stabilised with un-heated EWP and heat-treated EWP, three oil concentrations (5, 10 and 20%) were selected for each type of emulsions to investigate their microstructure. Figure 4.4 shows the CLSM microstructural features of the various emulsions prepared with 5, 10 and 20% oil. The oil droplets are stained red and the proteins stained green. The CLSM images obtained shows increasing oil droplet size with increasing oil concentration. The CLSM images confirmed the particle size results (Table 4.4) and visual appearance (Figure 4.2) that emulsions prepared with 5% oil had smaller droplets, whereas, emulsions containing 20% oil showed larger droplets in both UEWP and HEWP emulsions. Furthermore, the CLSM images shows clearly at 10 and 20% oil (especially at 20%) both droplets coalescence and protein bridging which are prevalent with lack of sufficient EWP needed to give full coverage to the oil droplets in the 1% (w/w) EWP emulsion system.

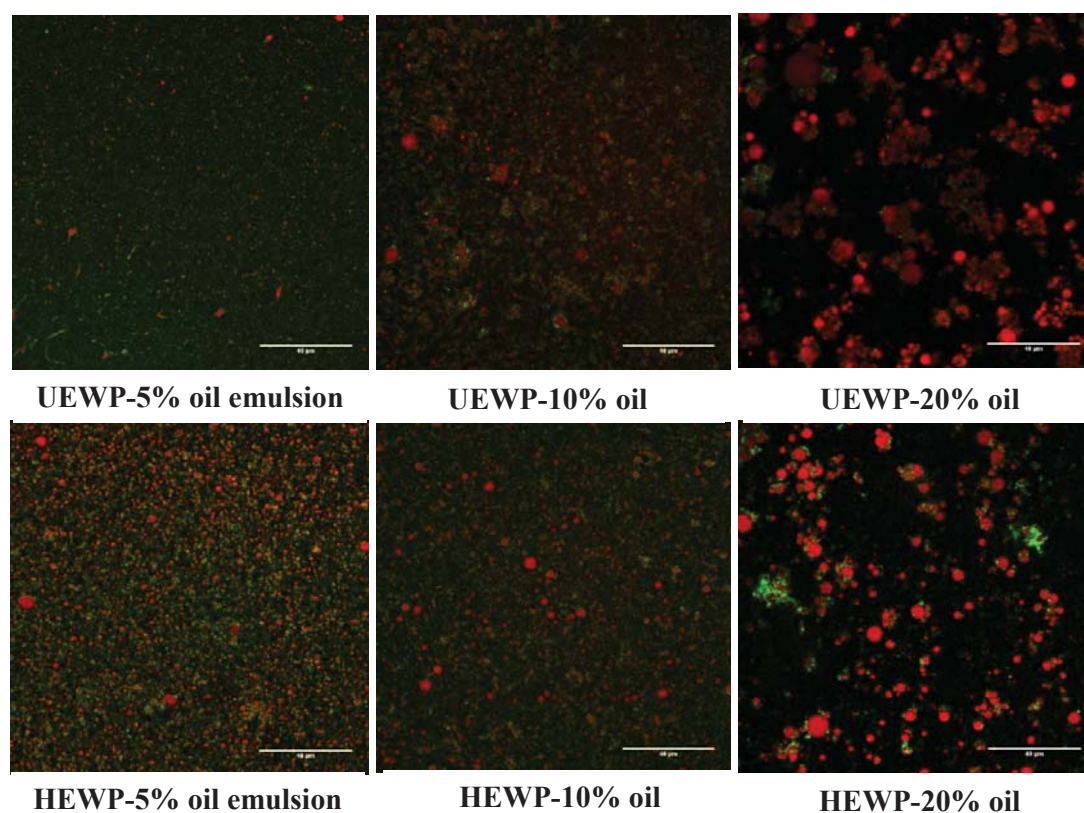


Figure 4.4: CLSM images of EWP-stabilized emulsions at 5, 10 and 20% oil concentrations. UEWP: Un-heated egg white protein and HEWP: heat-treated egg white protein. Each bar notes represents 40 μ m.

In summary, pre-heating of EWP prior homogenisation had no significant effect on the appearance and emulsifying properties of EWP-stabilised emulsions probably due to the heating time and protein concentrations used. Previous studies reported increased emulsifying property of spray dried EWP heated for a period of 10 days (Lechevalier et al., 2017a). Therefore, heating EWP solution for at least 1 day could denature the proteins such that the protein's flexibility, surface activity and hydrophobicity is increased probably leading to formation of less droplet aggregates and smaller droplet sizes. Nevertheless, EWP-stabilised emulsions were significantly affected by oil concentration, as droplet size increased with increasing oil concentrations. Interestingly, this study discovered less visible droplets aggregates/flocs can be produced at 5% oil concentration. Large droplet aggregates and flocs that have been found to occur in EWP-stabilised emulsions was attributed to bridging flocculation due to lack of sufficient proteins at higher oil concentrations (above 5%) to offer full coverage of the oil droplets and form sufficient thick interfacial layer.

4.4.2 Effect of oil and protein concentrations on formation of EWP aggregates during homogenisation

4.4.2.1 Visual observations

From the findings of the previous experiment described in Section 4.4.1, it was found that stable emulsions with a relatively small droplet size ($\sim 0.3 \mu\text{m}$) and little aggregates were produced at 5% oil concentration. On the other hand, the emulsions with large protein aggregates and large droplet size were produced when the concentration of oil was increased to 10% or higher. In this experiment, emulsions containing lower oil concentrations in a range of 1, 3, 5, 6, 7 and 10% were prepared with two different EWP concentrations (1 and 3%) to determine the concentration of oil and/or protein that causes the formation of EWP aggregates during the preparation of emulsions. As shown in Figure 4.5, at 1% EWP concentration little or no droplet aggregates were observed at 1, 3, 5 and 6% oil concentrations, while large EWP aggregates were observed from 7% oil and highest at 10% oil concentration. As previously explained in Section 4.4.1, the formation of less aggregates at $\leq 5\%$ oil can be attributed to availability of sufficient protein concentration which prevented droplet flocculation by bridging, while large aggregates formed at oil concentration above 6% was due to bridging flocculation caused by insufficient proteins available to fully cover the newly formed droplets and form sufficient dense layer around it (Sun & Gunasekaran, 2009).

However, when protein concentration increased to 3% only emulsions containing 1% oil contained little or no aggregates (Figure 4.6). This could be attributed to depletion flocculation caused by the presence of excess proteins in the continuous phase (Sourdet et al., 2003; Dickson, 2010). Depletion flocculation in an emulsion system occurs when the concentration of proteins in the continuous phase is above the required protein concentration for full coverage of the newly formed oil droplets. Therefore, the excess unadsorbed protein are excluded from the gap between the emulsion droplets due to osmotic pressure causing a net attraction between the emulsion droplets resulting in (Dickson & Golding, 1997; Dickson, 2010).

At both EWP concentrations, it was observed that aggregates/flocs formed increased with increasing oil concentration. The results of this study indicate that both oil and protein concentrations were responsible for the formation of aggregates in emulsions prepared with egg white liquid. To produce an EWP-stabilised emulsion with little or no visible

aggregates, it is advised that lower EWP concentration $\leq 1\%$ (w/w) be used. Nevertheless, even at 1% EWP concentration, the oil concentration is important and should be seriously considered, as oil concentrations above 6% resulted in aggregates formation which affected the appearance of the emulsion (as a result of visible large droplet aggregate and flocs) and could result in the blockage of the homogeniser as shown in Figure 4.7.

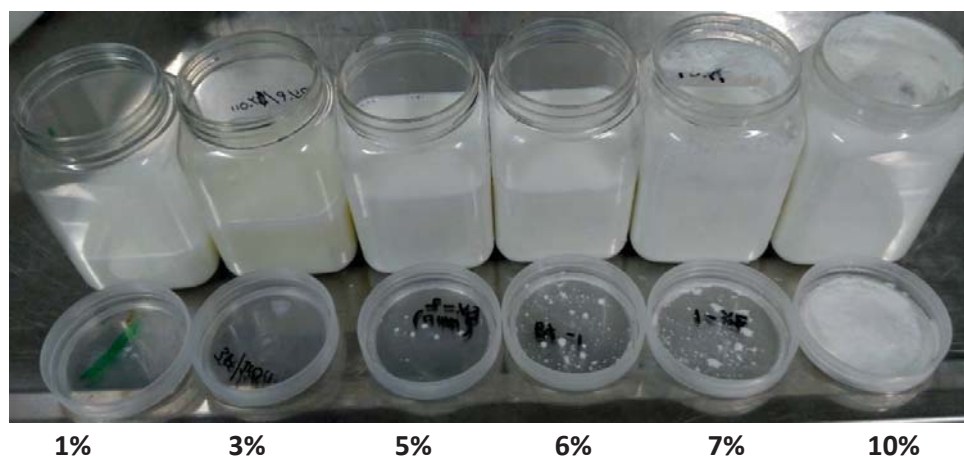


Figure 4.5: Emulsions prepared with 1% (w/w) EWP and different oil concentrations (1-10%). Protein aggregates increased as oil concentration increased from 1 to 10% (w/w).

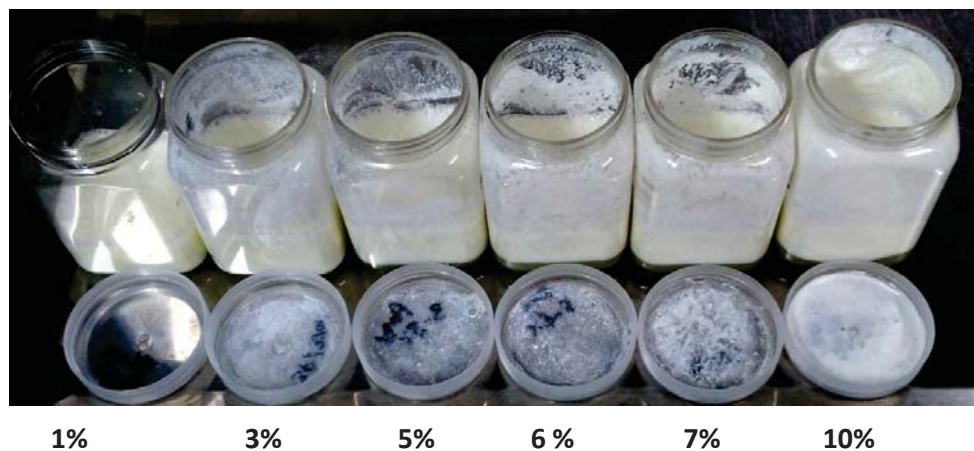


Figure 4.6: Emulsions prepared with 3% (w/w) EWP and different oil concentrations (1-10% w/w). Protein aggregates increased as oil concentration increased from 1 to 10% (w/w).



Figure 4.7: Photograph of droplets aggregates/flocs formed in the homogeniser during emulsion preparation.

4.4.2.2 Particle size and ζ -potential of emulsions

The results of Sauter mean diameter ($D_{3,2}$), volume mean diameter ($D_{4,3}$) and ζ -potential of EWP-stabilised emulsions are shown in Table 4.5. Generally, the volume mean diameter ($D_{4,3}$) indicates the presence of large droplet sizes while Sauter mean diameter ($D_{3,2}$) is identified with smaller droplet sizes. As shown in Table 4.5, there was a large difference between the $D_{3,2}$ and $D_{4,3}$ droplet sizes of 1% EWP-stabilised emulsions. $D_{3,2}$ droplet size ranged from 0.20 - 1.16 μm while $D_{4,3}$ ranged from 0.41 - 69.92 μm as oil concentration increased from 1 to 10%. A large difference between $D_{3,2}$ and $D_{4,3}$ signifies a polydisperse size distribution usually with more than one peak and when there is little difference, it signifies a monodisperse size distribution with one peak (Jafari, He, & Bhandari, 2006). Although droplet size increased with increasing oil concentration, $D_{3,2}$ showed significant difference ($p \geq 0.05$) in droplet size at 1 to 7% oil concentration and no significant difference at 1 to 5% oil concentration for $D_{4,3}$. However, droplet size at 10% was significantly higher than other oil concentrations with smaller droplets sizes observed below it.

The result of the droplet sizes confirmed the visual observation as little or no droplet aggregates were observed below 7% and largest droplet aggregates seen at 10%. As shown in Figure 4.8a, a monomodal size distribution was seen at 1%. Further addition of oil up to 6%, changed the particle size distribution (PSD) a bimodal size distribution with two peaks. At 7% oil, a multimodal size distribution was observed which could be responsible for the droplets aggregates seen in its visual appearance (Figure 4.5). However, at 10% the PSD

was bimodal shifting to the right towards larger size ranges. ζ -potential obtained for the 1% EWP-stabilised emulsions were greater than -28 mV and ranged from -28.9 to 37.7 mV with increasing oil concentration with 10% emulsion having the highest ζ -potential. Generally, a ζ -potential of ≥ 30 mV is needed to stabilise emulsions. Higher ζ -potential values ensure greater magnitude of repulsive interactions among emulsion oil droplets so that stability of the emulsions is assured (Li & Tan, 2007).

Table 4.5: Mean particle size ($D_{4,3}$ and $D_{3,2}$) and ζ -potential values of O/W emulsions containing different concentrations of canola oil (1,3, 5, 6, 7 and 10% w/w) stabilised by 1 and 3% (w/w) EWP

Protein (% w/w)	Oil (% w/w)	$D_{3,2}$ (μm)	$D_{4,3}$ (μm)	ζ -potential (mV)
1	1	$0.20 \pm 0.006^{\text{ef}}$	$0.41 \pm 0.004^{\text{h}}$	$-28.9 \pm 0.90^{\text{d}}$
	3	$0.26 \pm 0.006^{\text{ef}}$	$0.48 \pm 0.002^{\text{h}}$	$-32.3 \pm 1.11^{\text{c}}$
	5	$0.29 \pm 0.006^{\text{ef}}$	$0.56 \pm 0.009^{\text{h}}$	$-33.7 \pm 1.78^{\text{c}}$
	6	$0.36 \pm 0.007^{\text{c}}$	$0.97 \pm 0.032^{\text{g}}$	$-32.8 \pm 0.79^{\text{c}}$
	7	$0.38 \pm 0.006^{\text{c}}$	$1.82 \pm 0.948^{\text{f}}$	$-33.7 \pm 0.75^{\text{c}}$
	10	$1.61 \pm 0.090^{\text{d}}$	$69.92 \pm 5.768^{\text{d}}$	$-37.7 \pm 0.77^{\text{a}}$
3	1	$0.45 \pm 3.34^{\text{e}}$	$0.56 \pm 0.03^{\text{h}}$	$-23.7 \pm 1.00^{\text{f}}$
	3	$27.51 \pm 2.47^{\text{c}}$	$60.49 \pm 3.34^{\text{e}}$	$-25.1 \pm 0.37^{\text{e}}$
	5	$30.47 \pm 0.51^{\text{c}}$	$99.56 \pm 3.57^{\text{c}}$	$-30.1 \pm 1.14^{\text{cd}}$
	6	$36.16 \pm 3.87^{\text{b}}$	$108.81 \pm 4.71^{\text{c}}$	$-31.9 \pm 1.46^{\text{c}}$
	7	$63.07 \pm 10.44^{\text{a}}$	$162.01 \pm 15.81^{\text{a}}$	$-32.4 \pm 1.11^{\text{c}}$
	10	$58.87 \pm 4.64^{\text{a}}$	$121.21 \pm 10.29^{\text{b}}$	$-34.8 \pm 1.28^{\text{b}}$

Data represents mean $\pm$ standard deviation of two independent measurements (n=10). Different superscripts within a column are significantly difference at $p < 0.05$.

The same phenomenon was observed in 3% EWP-stabilised emulsions as mean particle size increased significantly as oil concentration increased. Nevertheless, $D_{4,3}$ showed emulsion particle size at 7% was higher than droplet size of 10% oil. Although it is not sure, why droplet 7% was higher than that of 10%, this might be due to the nature of emulsion samples having inhomogeneous large aggregates that caused random error, making the measurements to fluctuate. As shown in Figure 4.8b, all emulsions exhibited a monomodal size distribution with narrow PSD observed only at 1% oil while at higher oil concentrations,

the emulsions exhibited broad and similar PSD shifting towards larger sizes. This is not surprising as little or no droplets aggregates was formed when observed visually compared to the large droplet aggregates formed at above 1% (Figure 4.6). Additionally, droplet size obtained at 1% EWP was significantly smaller ($\sim 0.56 \mu\text{m}$) compared to the larger droplet sizes obtained at 10% oil concentrations ($69.92 \mu\text{m}$). The results of the PSD agreed with those of particle size results confirming smaller droplet sizes at 1% oil and larger droplets at higher oil concentration, with highest at 10%. ζ -potential of the 3% EWP-stabilized emulsions increased significantly from -23.7 to 34.8 mV as oil concentration increased from 1 to 10%.

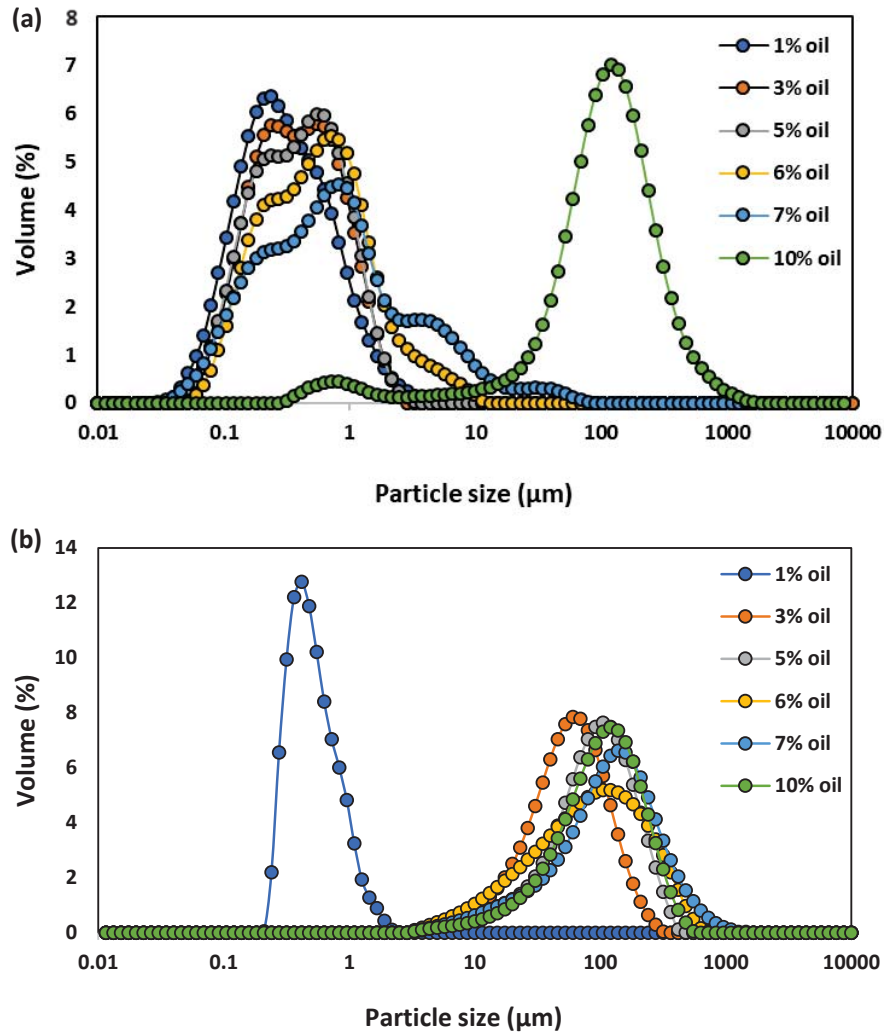


Figure 4.8: Particle size distributions of emulsions containing different oil concentrations (1, 3, 5, 6, 7 and 10% w/v) which were stabilized by 1% EWP (a) and 3% EWP (b)

In both 1% and 3% EWP-stabilized emulsions, droplet size increased with increasing oil concentration. These observations are similar to those reported by Sun & Gunasekaran (2009) who reported increased droplet size with increasing oil concentration in 0.2% whey protein isolate O/W emulsions prepared with different oil concentrations (5, 20 and 40% v/v). Guo & Mu (2011), also reported increased droplet size with increase in oil concentration in O/W emulsions prepared with 1% (v/v) sweet potato protein and different oil concentrations (5, 15, 25, 35 and 45% w/v). The formation of large droplet sizes in emulsions containing lower protein concentration and higher oil concentration can be expected due to lack of sufficient proteins to be adsorbed onto the surface of newly formed oil droplets, resulting in flocculation by bridging (Sun & Gunasekaran 2009; Guo & Mu, 2011). Additionally, increasing the oil concentration can promote collision frequency among the oil droplets because of increased viscosity of the continuous phase, subsequently increasing the rate of coalesce and flocculation (Sun & Gunasekaran).

Generally, it is expected for droplet size to decrease with increasing protein concentration. However, it was seen that emulsions containing 1% protein presented smaller particle sizes than those of 3% protein. Previous study by Romero et al. (2016) reported decreased droplet size with increasing oil concentrations in emulsions prepared with 65% sunflower oil and different egg white albumen concentrations (0.75, 1.5, 3.0 and 5%) at pH 3.0. Similar results were also reported in a study of O/W prepared with 15% sunflower/5% olive oil using different concentrations of whey protein isolates (1, 2 and 4% w/v) at ~pH 7 (Hebishy et al., 2015). The decrease in droplet size with increasing protein concentration observed in their study could be due to the high oil concentrations (650g/kg and 20%) used by these authors. Increasing the protein concentration in the continuous phase can promote the availability of proteins for protein adsorption unto the surface oil droplets which enhanced the formation of monolayer of multilayer around droplet surfaces preventing oil droplet coalescence (Dickson & Golding, 1997; Joshi et al., 2012).

However, increase in protein concentration in the continuous phase could decrease the effectiveness of protein adsorption unto the oil droplets due to increase in viscosity of the continuous phase. Increase in viscosity of the continuous phase can slow down the migration of the proteins unto the interface to be adsorbed by the oil droplets (Dagorn-Scaviner, Gueguen, & Lefebvre, 1987; Guo & Mu, 2011). The function of an emulsifier is to decrease the interfacial tension to enable the rapid adsorption of the proteins unto the

surface of the newly formed oil droplets with thick protective layer to stabilise the emulsions (Dickson & Golding, 1997; Sun & Gunasekaran, 2009). To attain an effective emulsion stabilisation, the concentration of an emulsifier is important (McClements, 2004; Sun & Gunasekaran, 2009). The results obtained in this study suggests smaller droplet sizes and less droplet aggregates are formed at 1% EWP concentrations.

4.4.2.3 Creaming stability

Emulsions are thermodynamically unstable and tend to break down with time due to several physical destabilization mechanisms, such as flocculation, phase separation, coalescence and Ostwald ripening (McClements, 2004). Emulsions stabilised by EWP were more stable against separation at 1% EWP concentration than at 3% (w/w) protein (Figure 4.9). Emulsions produced with 1% EWP were stable against separation visually even after 1-week storage at ambient temperature (20°C) at all different oil concentrations used. On the other hand, phase separation occurred rapidly soon after the emulsion preparation (i.e. after 1 hour) in 3% EWP emulsions containing different oil concentrations except for 1% oil. The reason for this separation could be attributed to their large droplet size as shown in Table 4.5. According to Stoke's law, the rate of phase separation in O/W is directly proportional to the size diameter of oil droplets. Thus, the larger the droplet size the higher the rate of phase separation of an emulsion (Joshi et al., 2012). Therefore, confirming droplet size to be an important factor in determining the stability of O/W emulsions against separation and creaming (McClements, 2004; Joshi et al., 2012). Furthermore, another mechanism for the instability at higher protein concentration could be due to depletion flocculation caused by excess non-adsorbing proteins present at the interface. As a consequence, oil droplets are attracted towards each other as the unadsorbed proteins are depleted from the narrow region between the oil droplets resulting in coalescence and flocculation (McClements, 2004; Sun & Gunasekaran, 2009).

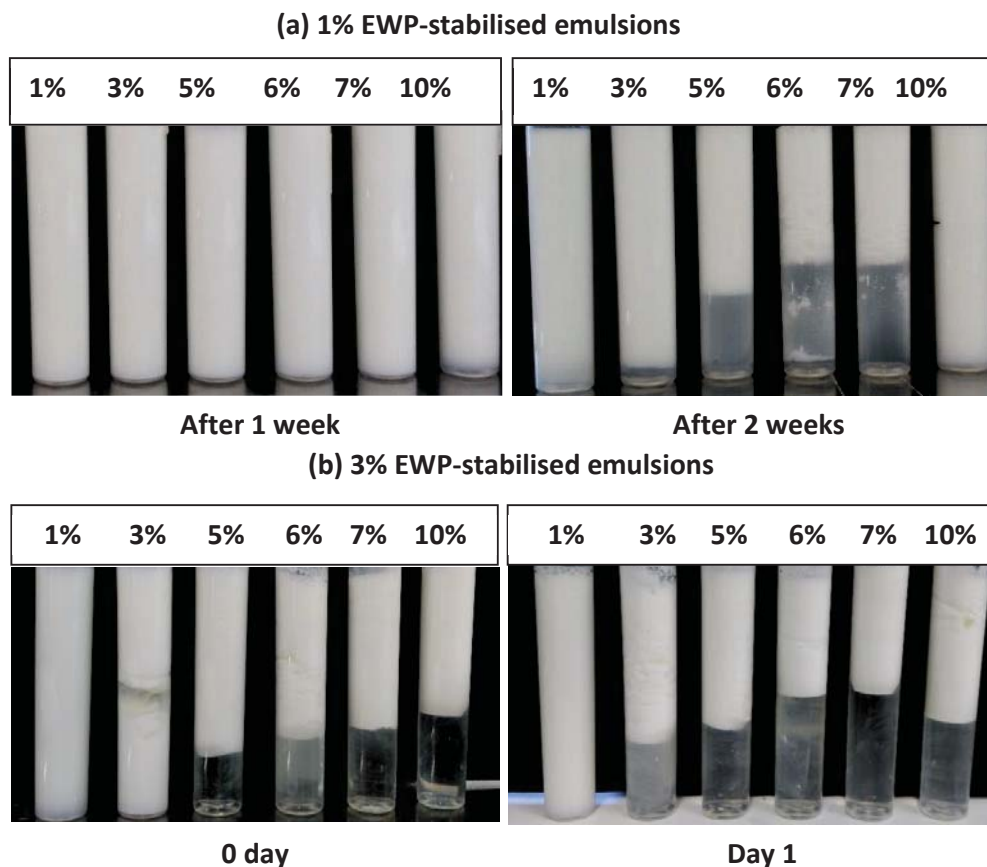


Figure 4.9: Creaming stability of emulsions stabilised containing different oil concentrations (1, 3, 5, 6, 7, and 10% w/w) stabilised by 1% EWP (a) and 3% EWP (b).

4.5 Conclusions

The effect of various factors such as pre-heating EWP prior homogenisation, oil concentration and protein concentration on the formation of aggregates and emulsifying properties of EWP-stabilised emulsions was investigated. Pre-heating of EWP solution at 60°C for 30 minutes to partially induce protein aggregation did not have any significant effect on the reduction of visible aggregates and droplet size of EWP emulsions. However, formation of visible aggregates and droplet size was found to increase significantly as oil concentration increased from 5 to 20% (w/w). Interestingly, smaller droplet size $\sim 0.32 \mu\text{m}$ and little or no visible aggregates was seen at 5% oil in 1% EWP. Smaller droplet size and less visible aggregates obtained at 5% oil was attributed to sufficient proteins available to form thick interfacial layer around the oil droplets. On the other hand, large aggregates and

droplet size at higher oil concentrations resulted due to bridging flocculation because of insufficient proteins available to form thick interfacial layers around the oil droplets.

Furthermore, the formation of visible aggregates and emulsifying property of emulsions prepared at lower oil concentration (1, 3, 5, 6, 7 and 10% w/w) and two protein concentrations (1 and 3% w/w) was studied. It was found that oil and protein concentration played an important role in affecting the aggregates formation, droplet size and emulsion stability against separation. The results indicated that visible aggregates and smaller droplet sizes were produced at oil concentrations below 7% in emulsions stabilised by 1% protein. Nevertheless, at 3% protein concentration very little visible aggregates and smaller droplets size ($\sim 0.56 \mu\text{m}$) was observed at only 1% oil concentration, but increasing significantly with increasing oil concentration. Emulsions stabilised by 1% protein produced smaller emulsion oil droplets and were more stable against separation than emulsions containing 3% protein. The instability at 3% protein concentration resulted from presence of excess unadsorbed proteins in the continuous phase which promoted droplet coalescence and flocculation by depletion.

Overall, the results of this study provided important information for the formulation of EWP-stabilised emulsions with little or no visible aggregates which to the best of the author's knowledge has not been reported anywhere else. Furthermore, the results indicated that formation of smaller emulsion droplets size and visible aggregates can be prepared with 1% EWP and oil concentrations below 7% or 3% EWP concentration and 1% oil.

Chapter 5. Influence of protein concentration on stability of EWP emulsions: Effects of heat treatment, freezing and thawing, ionic strength and pH

5.1 Abstract

This study investigated the influence of egg white protein (EWP) concentrations (0.1 - 2.0% w/w) on the formation and properties of emulsions prepared with 5% oil. The effects of heat treatment, freezing and thawing, ionic strength and pH on the stability of emulsions were also investigated by analysing the droplet size, ζ -potential, microstructure of emulsions. Little visible aggregates were produced in emulsions prepared with 0.1-1% EWP. However, the aggregates increased with increased protein concentration. All the emulsions showed no signs of separation and were stable even after 5 days. It was found that the mean droplet size of emulsions increased significantly from 242.1 to 703.7 nm as protein concentration increased from 0.1 to 2%. Although, smallest droplet size was found at 0.3% rather than at 0.1% EWP concentration. Thus, indicating individual droplets were sufficiently covered at 0.3% EWP concentration. ζ -potential of the emulsion droplets was not affected by an increase in protein concentration. Heat treatment at temperatures 60, 65, 70, 80 and 90°C and holding time (0 and 30 minutes) had no significant effect on emulsion droplet size in all the protein concentrations used when compared to control emulsion (without heat treatment). Phase separation was observed in all the emulsions prepared with the various protein concentrations after freezing at -20°C for 24 hours and thawing at 4 and 20°C. Furthermore, extensive flocculation was observed at ionic strength of 5-100 mM CaCl_2 and 50-600 mM NaCl, especially at low protein concentrations (0.1- 0.8%). Addition of excess protein (1-2%) significantly improved the stability of the emulsions against salt-induced flocculation. Emulsions at acidic pH (pH 2.0) and alkaline pH (8-10) were found to remain stable to droplet aggregation, but at pH 4-5 close to the isoelectric point (pI) of EWP, extensive droplet aggregation was observed to occur as expected due to weak electrostatic repulsive forces. This study showed that the properties and formation of emulsions stabilised by EWP were significantly affected by protein concentration. The results provide an important information in the use of EWP as an emulsifier for the design and production of stable EWP emulsions.

5.2 Introduction

A majority of oil-in-water (O/W) food emulsion products (e.g. yoghurt, dips, mayonnaise, and some beverages) are prepared with natural emulsifying agents, such as proteins (Singh, Ye, & Horne, 2009; Amine, Dreher, Helgason, & Tadros, 2014). Proteins with amphiphilic character have surface active properties, thus enabling their use as emulsifiers to form and stabilise O/W emulsions (Dickson & Golding, 1997; Norde, 2003; McClements, 2004). During emulsification, proteins diffuse into the O/W interface and adsorb rapidly onto the surface of the newly formed droplets to lower the interfacial tension and prevent droplet coalescence by providing a protective coverage around the droplets (Walstra, 2003; McClements, 2004). The adsorbed proteins can stabilise droplets against coalescence and flocculation during storage by steric and/or electrostatic repulsive forces (Dickson, 1992; Wilde, Mackie, Husband, Gunning, & Morris, 2004; Tadros, 2013).

A number of studies have reported the properties of O/W emulsions formed from various protein sources: plant proteins like soy, potato and pea (Gharsallaoui, Cases, Chambin, & Saurel, 2009; Guo & Mu, 2011); and milk proteins like whey and caseins (Srinivasan, Singh, & Munro, 1999; San Martin-Gonzalez, Rouch, & Harte, 2009; Hebishy et al., 2015). Egg white comprising mainly of proteins and water has also been used in some studies to investigate the formation and properties of egg white protein-stabilised emulsions (Mine et al., 1991; Kudryashova, Visser, van Hoek, & de Jongh, 2007; Romero, Perez-Puyana, Marchal, Choplin, & Guerrero, 2017). Its major protein components, such as ovalbumin, conalbumin, lysozyme and ovomucoid, are responsible for its functional properties (Drakos & Kiosseoglou, 2006).

An emulsion system is thermodynamically unstable and its instability results from several destabilisation mechanisms, such as creaming, coalescence, flocculation and Ostwald ripening (Damodaran, 2005; McClements, 2005). The stability of protein-stabilised emulsions is also strongly dependent on some variables and environmental conditions like protein type, protein concentration, pH, ionic strength, heat treatment and freezing and thawing, which in turn determines the physicochemical properties of emulsions, such as oil droplet size, microstructure, rheology, interfacial properties, creaming and stability (Sun & Gunasekaran, 2009; Guo & Mu, 2011). Previous studies have investigated the effect of protein concentration on the rheology, physical stability, droplet size and emulsifying ability and stability of emulsions prepared with egg white protein (EWP). For instance,

Mine et al. (1991) determined the emulsifying ability (EA) and emulsifying capacity (EC) of emulsions containing 0.1-5.0% concentrations of ovalbumin and various amounts of soya bean oil (12.5, 25 and 50% w/v). Padala, Williams, & Philips (2009) investigated emulsifying properties of 20% limonene O/W emulsions using various concentrations of egg white powder (0.1-1.0% w/w) prepared at pH 3.5 and 7.5. In a recent study reported by Romero et al. (2017), O/W emulsions containing 65% sunflower oil were prepared with different egg white powder concentrations (0.75-5.0%) at pH 3.0. The authors reported increased EA, EC and decreased emulsion droplet size with increasing EWP concentrations.

Protein-stabilised emulsions are susceptible to heat treatment, ionic strength and pH change (Kulmyrzaev, Chanamai, & McClements, 2000; Liang et al., 2017). Generally, the oil droplet size of protein-stabilised emulsions increases after heat treatment and the degree of coagulation and aggregation of oil droplets depends on the heating time and temperature (Liang, Wong, Pham, & Tan, 2016). Addition of salts also influences emulsion stability and its effect depends on salt type, its valence and size and concentration (McClements, 1999). Generally, droplet size and droplet aggregation increase with increase in ionic strength. Chang et al. (2016) reported that at 50 mM NaCl, no aggregation occurred in egg white protein emulsions, but at 100 mM, the emulsions suffered major aggregation and flocculation.

The distribution and nature of electrical charges on a protein molecule also play an important role on the stability of emulsions which can be significantly affected by pH change. In general, emulsions stabilised by proteins tend to flocculate at pH close to the isoelectric point of protein molecules because electrostatic repulsions of oil droplets become weak to overcome attractive interactions resulting in strong droplet aggregation/flocculation and decreased emulsion stability (Gu, Decker, & McClements, 2005; Drakos & Kiosseoglou, 2006). Previous studies have indicated stable egg white emulsions can be prepared at acidic pH 3 and 4 rather than neutral pH (Mine et al., 1991; Drakos & Kiosseoglou, 2006; Chang et al., 2016; Niu et al., 2017). According to the authors, at acidic pH, hydrophobic amino acid groups and charged amino acid groups are more exposed to the surface; they undergo partial unfolding and become more flexible (Raghavan & Kristinsson, 2007).

In Chapter 4, the effects of heat treatment, protein concentration and oil concentration on the reduction of aggregates formed in emulsions prepared with EWP were investigated. It was found that emulsions with little or no aggregates could be produced when prepared with either 1% EWP and <6% oil or 3% EWP and 1% oil concentration. On the other hand, heat treatment of EWL (1% w/w) at 60°C for 30 minutes prior to homogenisation had no significant influence in reducing aggregates in emulsions containing 10% oil. Therefore, in this study, the influence of lower EWP concentrations (0.1, 0.3, 0.5, 0.8, 1 and 2% w/w) on the properties and stability of emulsions containing 5% oil prepared at pH 8.3 was investigated. In this study, 5% oil concentration was chosen throughout because of the little or no aggregates that were formed in emulsions stabilised by 1% EWP as shown in Chapter 4. Furthermore, the effects of heat treatments, freezing and thawing, pH and ionic strength on the properties and stability of the emulsions prepared with 5% oil and various EWP concentrations were investigated. The properties of the emulsions were analysed by particle size, zeta potential and microstructure measurements.

5.3 Materials and Methods

5.3.1 Materials

Pasteurised egg white liquid (containing 10% w/w protein) (Zeagold Foods, New Zealand) and canola oil (Budget, Safeway Traders Ltd, New Zealand) were purchased from a local supermarket. Hydrochloric acid (HCl), sodium hydroxide (NaOH), sodium chloride (NaCl), calcium chloride (CaCl₂), and sodium azide (NaN₃) were purchased from Thermo Fisher Scientific Inc., New Zealand.

5.3.2 Preparation of emulsions

To compare the properties of emulsions prepared with various protein concentrations, EWL containing 10% (w/w) protein concentration was diluted with distilled water to protein concentrations of 0.1, 0.3, 0.5, 0.8, 1.0 and 2.0% (w/w). The original pH of EWL (~pH 9.0) was used for emulsifications without any pH adjustment. Then, oil was mixed with each protein solutions at a weight ratio of 5:95 and pre-homogenised using an Ultra-Turrax blender (IKA T25, Staufen, Germany) at 13,000 rpm for 2 minutes to form coarse emulsions. The coarse emulsions obtained were further homogenised to produce fine emulsions using an APV 2000 2-stage high pressure value homogeniser (APV Manufacturing, Poland) 4 times at pressure level of 500/50 MPa as previously described in Section 4.3.2.1. After preparation, the emulsions were stored at 4°C until further analysis.

5.3.3 Effects of environmental conditions on emulsions

The effects of heat treatment, freezing and thawing, pH changes and ionic strength on the stability of EWP-stabilised emulsions containing different protein concentrations were studied.

- The effect of thermal treatment on stability of emulsions was examined by placing 10 ml emulsion in glass test tubes and incubating in a water bath at different temperatures (60, 65, 70, 80 and 90°C) at different heating times (0 and 30 minutes). After heating, the emulsions were immediately placed in an ice water bath to cool them down.
- The effect of freezing and thawing was studied by freezing the emulsion samples at -20°C and then thawing at 4°C and 20°C after 24 hours.
- The influence of ionic strength on emulsion stability was determined by adding sodium chloride (NaCl) and calcium chloride (CaCl₂) salts to the emulsions. The emulsions were mixed with different concentrations of salt (0, 10, 50, 100 mM for CaCl₂ and 0, 50, 100, 200, 400 and 600 mM for NaCl) (see Tables 5.1 and 5.2), then vortexed to ensure uniform mixing.
- The effect of pH on emulsion stability was investigated from emulsions containing 1% protein and 5% oil by adjusting the original pH of the emulsions (8.9) to different pH levels (2, 3, 4, 5, 6, 7, 8, 9 and 10) using different concentrations of HCl and NaOH solutions.

Table 5.1: Addition of CaCl₂ at different concentrations to EWP-stabilised emulsions containing 5% oil (w/w) and various protein concentrations (0.1, 0.3, 0.5, 0.8, 1.0 and 2.0% w/w)

CaCl ₂ (mM)	CaCl ₂ (g)	Emulsion (g)	Total (g)
0	0	10	10
5	0.007	9.993	10
10	0.015	9.985	10
50	0.074	9.926	10
100	0.147	9.853	10

Table 5.2: Addition of NaCl at different concentrations to EWP-stabilised emulsions containing 5% oil (w/w) and various protein concentrations (0.1, 0.3, 0.5, 0.8, 1.0 and 2.0% w/w)

NaCl (mM)	NaCl (g)	Emulsion (g)	Total (g)
0	0	10	10
50	0.029	9.971	10
100	0.058	9.942	10
200	0.117	9.883	10
400	0.234	9.766	10
600	0.351	9.649	10

5.3.4 Particle size and zeta (ζ) potential measurements

The particle size and size distribution of emulsions were determined by dynamic light scattering (DLS) using a Zetasizer Nano ZS90 instrument (Malvern Instruments Ltd., Worcestershire, UK) which was equipped with a helium/neon laser at a 633 nm wavelength and analysed at a scattering angle of 173°. Emulsion samples were placed in a DTS1060 folded capillary cell (Malvern Instruments Ltd., Worcestershire, UK) which was loaded into the instrument without dilution. The emulsion samples were equilibrated for 60 seconds at 25°C in the instrument before data reading. Particle size results are reported as Z-average mean diameter, which is the intensity-weighted mean hydrodynamic size of the particles. For some samples with large aggregates, a Malvern Mastersizer (Malvern Instruments Ltd., Worcestershire, UK) was used as previously described in Section 4.3.2.2 and particle size is reported as surface mean diameter ($D_{3,2}$). The electrical charge (ζ -potential) of the emulsions was measured using a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd, Worcestershire, U.K) as previously described in Section 3.3.3. Prior to measurement, the emulsion samples were diluted with distilled water to a ratio of 1:100. All measurements were carried out in at least duplicate on two independent samples.

5.3.5 Confocal laser scanning microscopy

The microstructure of the emulsions was determined by confocal laser scanning microscope (CLSM) as previously described in Section 4.3.2.3.

5.3.6 Statistical analysis

All experiments and sample analyses were carried out in at least duplicate. Results are presented as mean and standard deviation. Data were analysed statistically by analysis of variance (ANOVA) using Minitab statistical software version 17 (Minitab Inc., USA). Significant difference between means was analysed by Turkey's HSD method at a confidence level of 95% ($p < 0.05$).

5.4 Results and Discussion

5.4.1 Effect of EWP concentration on particle size and size distribution

The effect of protein concentration on the properties of emulsions stabilised with various protein concentrations (0.1, 0.3, 0.5, 0.8, 1.0 and 2% w/w) and 5% (w/w) oil concentration prepared at pH 8.3 was investigated. From the visual observation (photographs not shown, as it was hard to spot the difference visually), emulsions prepared at 0.1% to 1.0% showed very little visible aggregates. However, aggregates increased with increased in protein concentration. Visually, the emulsions were similar to emulsions prepared with 1% EWP and oil concentrations less than 7% as reported in previous experiments conducted in Chapter 4 (Section 4.4.2; Figure 4.5). At 2%, the aggregates were larger than those obtained at lower protein concentrations but much smaller to aggregates formed when emulsions were prepared with 1% EWP and oil concentration $>$ than 6% (Chapter 4; Figure 4.5). From the results of previous experiments (Chapter 4; Section 4.4.2), it was observed that little or no aggregates were produced in emulsions prepared with 1% EWP and $<7\%$ oil and 3% EWP and 1% oil. Thereby indicating protein and oil concentration significantly affected the appearance of emulsions stabilised by EWP. Furthermore, the emulsions prepared with 0.1 to 2% EWP concentration showed no sign of separation on the day of preparation and were stable against separation even after five days. The results of mean particle size expressed as Z-average and particle size distribution of emulsions are shown in Figures 5.1a and b, respectively.

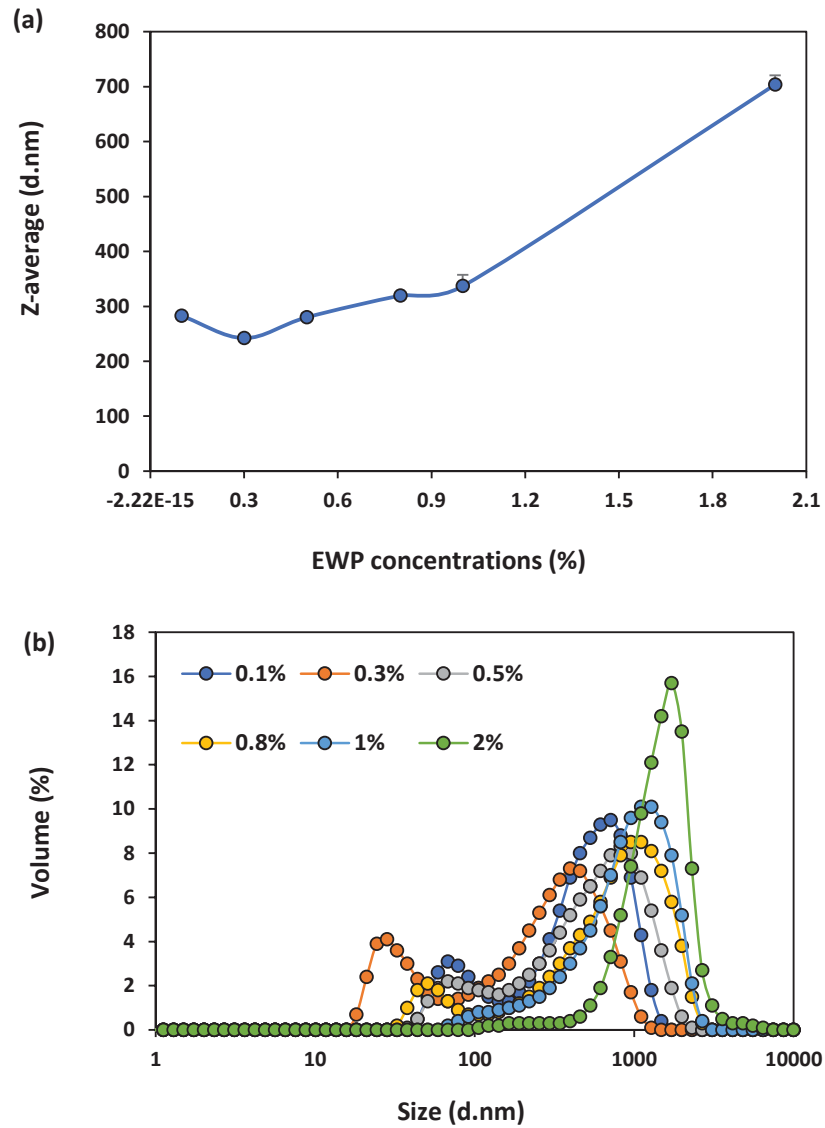


Figure 5.1: (a) Z-average and (b) particle size distributions of O/W emulsions prepared with 5% (w/w) oil and various protein concentrations (0.1, 0.3, 0.5, 0.8, 1 and 2% w/w). Data points represents mean $\pm$ standard deviation (n=6).

The results obtained show that protein concentration significantly affected particle size ($p < 0.05$), as particle sizes increased from 242.1 to 703.7 nm in diameter. The results also show increasing Z-average with increase in protein concentration except for droplet size at 0.3% EWP concentration which had the mean droplet size (242.1 nm) smaller than at 0.1% (282.8 nm). The small droplet size obtained at 0.3% protein, could indicate that the EWP concentration was sufficient enough to cover the emulsion oil droplets and form a sufficient dense layer to stabilise the oil droplets but was not enough to induce depletion flocculation (Sanchez & Patino, 2005). The results obtained in this study are not in agreement with those

reported by other researchers for emulsions stabilised with egg white proteins using various protein concentrations (Mine et al., 1991; Padala et al., 2009; Romero et al., 2017), as particle size was observed to reduce with increasing protein concentrations. Similar decrease in particle size with increasing protein concentration has also been reported in emulsions stabilised with proteins from different sources as follows: whey protein 0.37-2.93% w/w (Lizarraga et al., 2008) and 1, 2 & 4% w/w, (Hebishy et al., 2015); sweet potato protein 0.1-2% w/v (Guo & Mu, 2011); soy protein and sodium caseinate 0.5, 1 & 2% w/w (Palazolo, Sobral, & Wagner, 2011).

The reason for the difference obtained in this study with works of other researchers especially studies carried out using egg white protein could be attributed to differences in the properties of EWP from different sources (e.g. powder or liquid; pasteurized or unpasteurized) as well as the physicochemical properties of emulsions (e.g. pH, type of oil and oil concentration). For instance, in this study, emulsions were prepared at pH 8.3 using 5% (w/w) canola oil and various EWP concentrations. Whereas, in other studies that reported decrease in particle size with increasing protein concentration, pH used ranged from pH 3.0-7.5; oil concentration was above 10%. Padala et al. (2009) reported decreased particle size with increasing EWP concentration in emulsions containing 20% limonene oil and various EWP concentrations (0.1-1.0% w/w) prepared at pH 3.5 and 7.5. Similar results were also reported in O/W emulsions stabilised by EWP containing 65% sunflower oil and various EWP concentrations (0.75-5.0%) at pH 3.0 (Romero et al., 2017). However, previous studies have reported that the emulsifying properties of egg white protein is considered poor when compared to other proteins (such as whey, casein and soy). They reported that the hydrophobic amino acids groups of EWP especially at neutral and alkaline pH (pH 7-11) are deeply buried in the protein structure, thus, not available during emulsification (Drakos & Kiosseoglou, 2006; Chang et al. 2016; 2017). Nevertheless, at acidic pH (pH 3.0 to 4.5), stable EWP emulsions can be prepared. At acidic pH, the proteins have an overall net positive charge which is expected to form electrostatic complexes, hence stable emulsion is produced (Mine et al., 1991). Secondly, at acidic pH, the hydrophobic amino acid groups and charged amino acid groups are more exposed to the surface; they undergo partial unfolding and become more flexible, yet with little changes in their structure. Thus, they are able to reorient and rapidly adsorb at the O/W interface (Drakos & Kiosseoglou, 2006; Chang et al., 2016).

However, similar results to those obtained in this study were reported in microfibrillated cellulose (MFC)-stabilised emulsions prepared with 10% (w/w) soybean oil and various MFC concentrations (0.05 to 0.70% w/w) at pH 7.0 (Winuprasith & Supphantharika, 2015). They showed increasing emulsion droplet size with increasing emulsifier concentration and reported that although it is expected for droplet size to decrease with increasing emulsifier concentration, in some cases the reverse is the case. A high emulsifier concentration could result in an increase in viscosity of the continuous phase which reduces the oil droplet movement during homogenisation process and their speed of adsorption at the interface (Winuprasith & Supphantharika, 2015). Another theory was reported by Dickson & Golding (1997) who investigated the effect of unadsorbed sodium caseinate on depletion flocculation in O/W emulsions prepared with various protein concentrations (1.6-6.0% w/w). They observed that an emulsifier concentration required for complete surface coverage of emulsion droplets is one that causes no flocculation, aggregation or coalescence. At above 2% protein, droplet aggregation and flocculation could be observed, hence they concluded that an excessive amount of emulsifier in the continuous phase can induce osmotic pressure gradient which causes a net attraction between emulsion droplets leading to flocculation by depletion. Yao et al. (2013) and Gao et al. (2017) found that the stability of O/W emulsions prepared with gum arabic (GA) or β -lactoglobulin (β -lg) required an optimal emulsifier concentration and that excessive concentration (above 5% GA or 5 mg/ml β -lg fibrils) resulted in depletion flocculation, droplet aggregation and coalescence, leading to large droplet size. In summary, emulsions prepared with lower EWP concentrations (0.1% to 1%) showed little or no visible aggregates. However, at 2% visible aggregates more than those obtained prepared with 0.1 to 1% EWP. Thus, formation of visible aggregates at 5% oil could be observed at 2% EWP concentration. Secondly, droplet size was significantly affected by the protein concentration of EWP, since the droplet size increased with increasing protein concentration.

As shown in Figure 5.1b, a bimodal particle size distribution with two peaks was observed for the emulsions prepared at 0.3-0.8% EWP concentrations, with a relatively large volume of smaller droplet sizes at 0.3% EWP. When the protein concentration increased to 1 and 2%, a monomodal particle size distribution was observed with droplet size shifting to larger droplet sizes.

5.4.2 Effect of EWP concentration on ζ -potential

As shown in Figure 5.2, ζ -potential values of emulsions prepared at different EWP concentrations were found to range from -35.3 to 39.2 mV. Additionally, ζ -potential measured from emulsions were not significantly affected by differences in the EWP concentrations used. ζ -potential represents the magnitude of repulsive force acting on oil droplets which plays an important role in maintaining the stability of emulsions against droplet interaction and aggregation (Li & Tian, 2007). In general, ζ -potential values greater than +30 mV or -30 mV are required to protect emulsion oil droplets from aggregation and coalescence (Li & Tian, 2007).

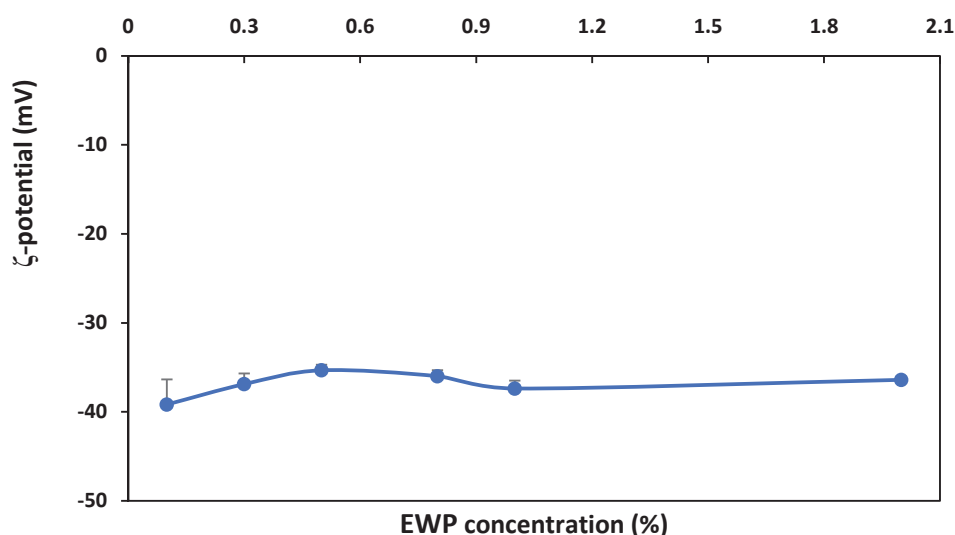


Figure 5.2: ζ -potential of O/W emulsions prepared with 5% (w/w) oil and various protein concentrations (0.1, 0.3, 0.5, 0.8, 1 and 2% w/w). Data points represent mean $\pm$ standard deviation (n=6).

5.4.3 Effect of thermal treatment on protein concentration of EWP emulsions

5.4.3.1 Particle size

Emulsion-based products are generally subjected to heat treatment during production to increase their microbial shelf stability (Chang et al., 2016). Therefore, the heat stability of emulsions containing different protein concentrations (0.1-2% EWP) was investigated by heating the emulsion samples at 60, 65, 70, 80 and 90°C for 0 and 30 minutes. The results of the particle size of emulsions measured before and after heat treatment for 0 and 30

minutes are illustrated in Figure 5.3. It seemed that there was no significant effect ($p < 0.05$) of temperature and holding time on the particle size of all emulsions containing 0.1%, 0.3%, 0.5% and 1% when compared to their control samples without heat treatment. However, at 0.8% protein significant difference in particle size was observed at 60 and 70°C, while at 2%, significant difference was observed at 60, 65 and 70°C. It should be noted that no visible sign of droplet aggregation or flocculation that could be attributed to the heat treatment was observed, regardless of the EWP concentrations (i.e. 0.1-2%) used in this study. It is generally expected that after heat treatment, the particle size of protein-stabilised emulsions becomes larger and droplet aggregation and/ or flocculation can occur depending on the temperature and holding time (McSweeney et al., 2004; Liang, Patel, Matia-Merino, Ye, & Golding, 2013). Thermal treatment of globular proteins (e.g. egg white proteins) induce them to unfold their conformational structure during heating, causing the exposure of non-polar and sulfhydryl amino acids from the interior of the protein molecules with increased surface hydrophobicity (Singh, 2011; Liang et al., 2017).

5.4.3.2 Confocal laser scanning microscopy

To better understand the effect of thermal treatment on emulsions containing various EWP concentrations, the emulsion microstructure was examined using CLSM. The CLSM images as shown in Figure 5.4 revealed no noticeable differences in the particle size of oil droplets between and within the emulsions samples at various temperatures and holding time. This was in agreement with the results of mean particle size shown in Figure 5.3. However, it should also be mentioned that the Z-average of 2% EWP emulsion samples measured to be relatively larger in size compared with the other samples could not be observed clearly in the CLSM image. The images also show there was no significant change in droplet size with increase in temperature and holding time in all emulsions containing different protein concentrations.

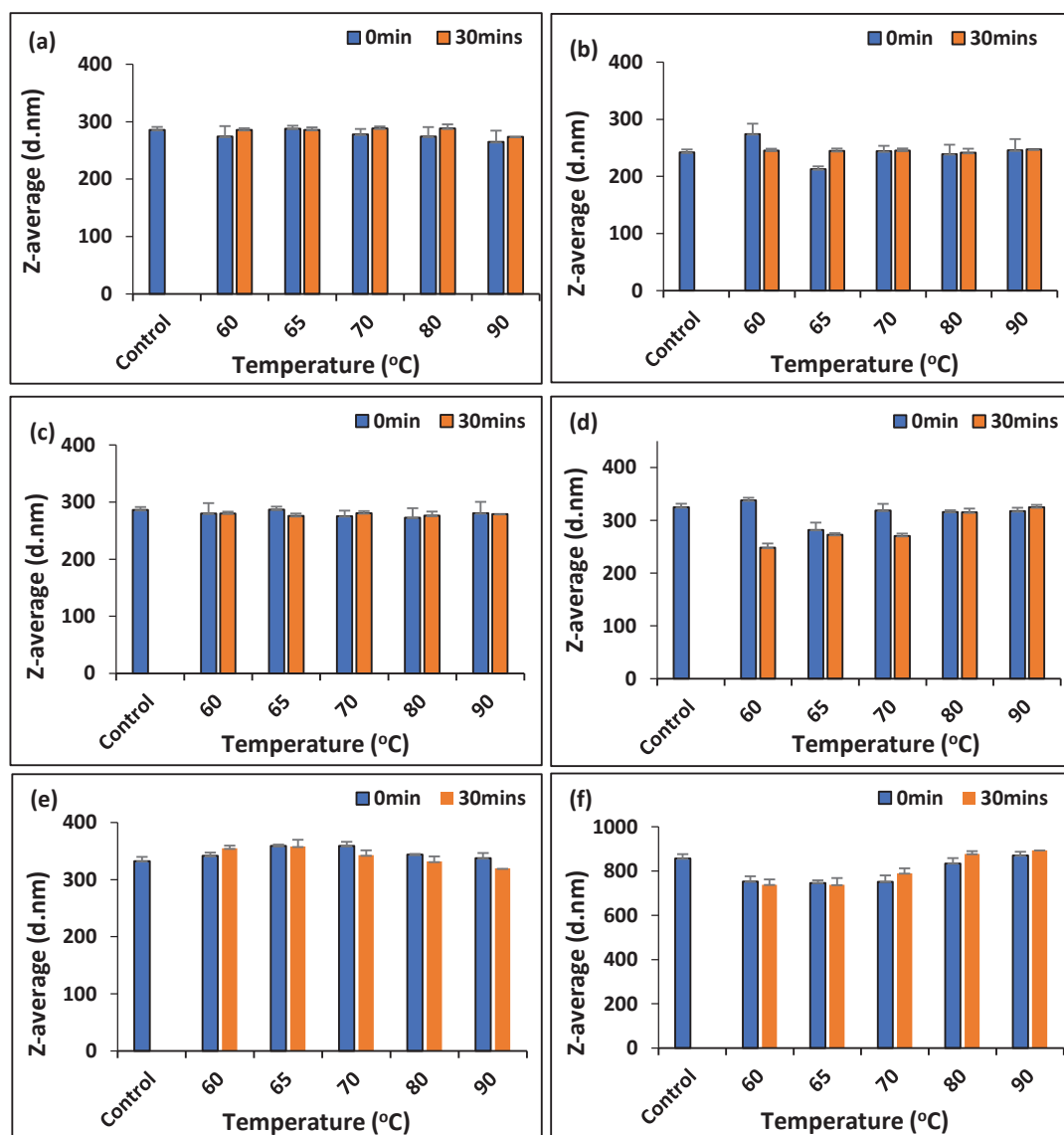


Figure 5.3: Effects of heat treatment of temperature (60, 65, 70, 80 and 90°C) and holding time (0 and 30 minutes) on the mean particle size (Z-average) of emulsions prepared with different protein concentrations. Control indicates emulsion samples without heat treatment (a) 0.1% (b) 0.3% (c) 0.5% (d) 0.8% (e) 1% and (f) 2%. Each data point indicates mean $\pm$ SD for n=6.

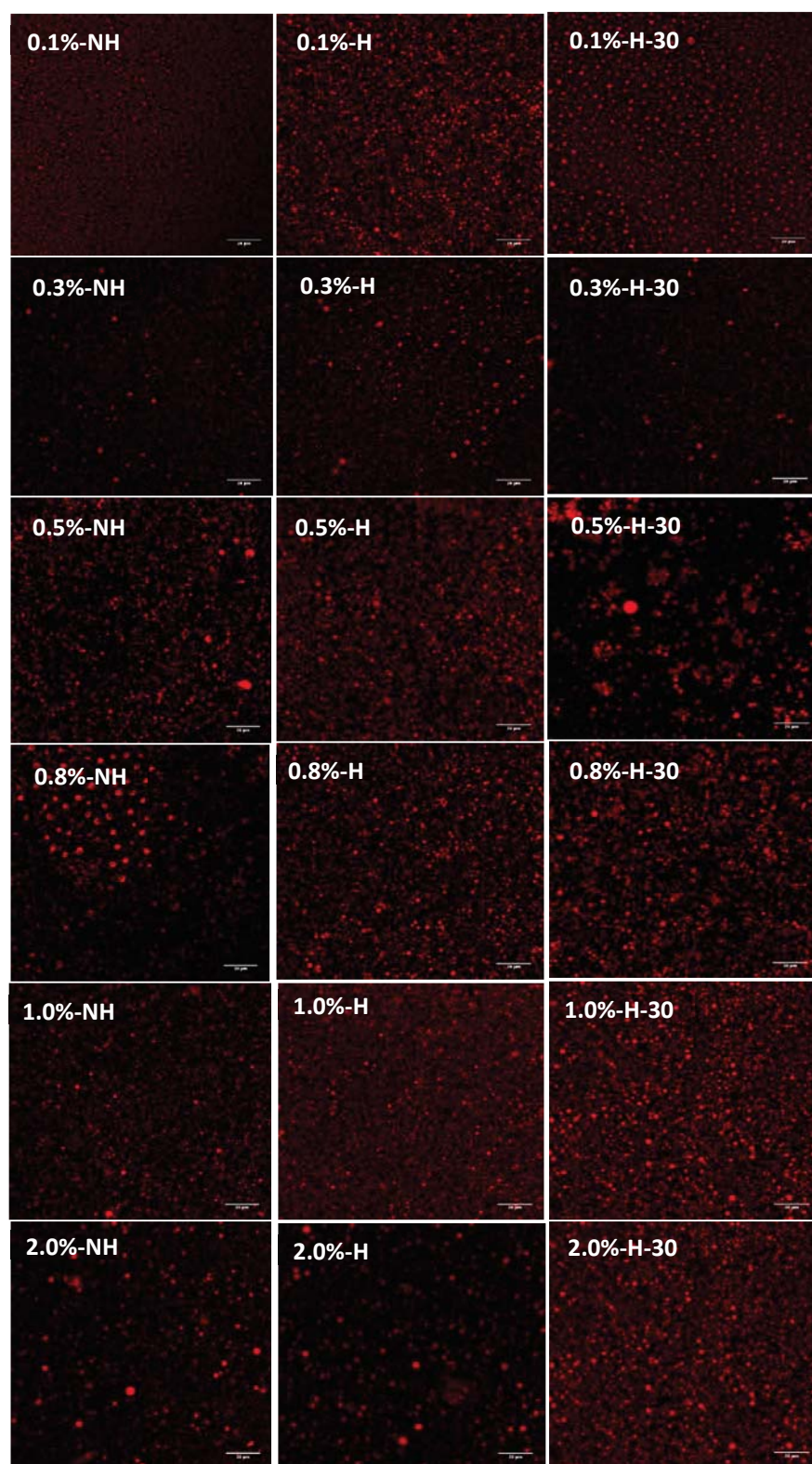


Figure 5.4: Confocal images of O/W emulsions prepared with 5% w/v oil and various EWP concentrations (0.1, 0.3, 0.5, 0.8, 1 and 2% w/w) taken before and after heat treatment at 90°C for 0 or 30 minutes. NH=no heat treatment (i.e. control samples), H= heat treatment for 0 minute and H-30= heat treatment for 30 minutes. The scale bar inserted represents 20 μm .

5.4.3 Effect of freezing and thawing

Some food products containing emulsions are frozen as part of their production process (e.g. frozen cocktails and ice cream) or to extend their shelf life (e.g. sauces and some beverages) (Ghosh & Coupland, 2008; Thanasukam, Pongsawatmanit, & McClements, 2004). However, thawing after freezing destabilizes emulsions resulting in partial coalescence which could cause phase separation into the aqueous and oil phase layer (McClements, 1999; Walstra, 2003). The stability of EWP-stabilised emulsions containing different protein concentrations (0.1-2% w/w), which were frozen at -20°C for 24 hours and then thawed at 4°C and 20°C for 12 hours to melt, was investigated. Upon thawing at 4°C and 20°C respectively, the emulsions irrespective of the protein concentration (0.1-2.0% w/w) became unstable, separating into a rich opaque droplet layer floating at the top and a transparent or slightly turbid aqueous layer at the bottom. This suggests EWP-stabilised emulsions are destabilized upon freezing and thawing. The pictures of the emulsion samples taken before and after thawing are shown in Figure 5.5.

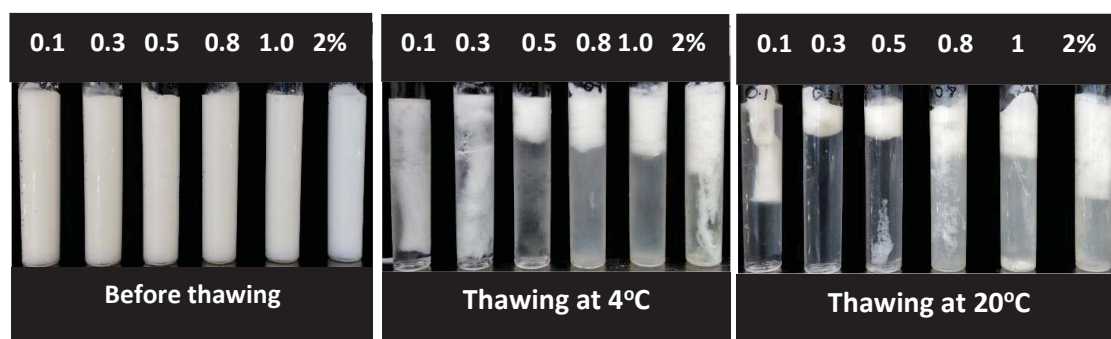


Figure 5.5: Photographs of phase separation of emulsions stabilised by EWP containing various protein concentration (0.1, 0.3, 0.5, 0.8, 1.0 and 2.0% w/w) and 5% oil (w/w) after freezing at -20°C for 24 hours and thawing at 4°C and 20°C, respectively.

Several factors could be responsible for the destabilisation of frozen and thawed emulsions. Firstly, crystallisation of the aqueous phase forcing droplets closer together to form droplet-droplet interactions (Hartel, 2001; Thanasukam et al., 2014). Secondly, freezing of the aqueous phase reduced the available free liquid water needed fully to hydrate the emulsifiers at the droplet surface encouraging droplet-droplet interactions (Carvajal, MacDonald, & Lanier, 1999; Walstra, 1999). Thirdly, at low temperatures cold denaturation of proteins occurs leading to loss of the functionality of protein as emulsifier

when thawed (Carvajal et al., 1999; McClements, 2002). Lastly, the amount of emulsifier needed to cover the droplets completely is reduced because some of the emulsifiers adsorb to the surface of the ice crystals (Hillgren, Lindgren, & Alden, 2002).

5.4.4 Effects of salt type and ionic strength

5.4.4.1 Particle size and size distribution

A number of emulsion-based products such as sauces and soups are supplemented with minerals from different types of salt. It is important to understand the effect of salt on the stability of emulsions stabilised by EWP. For this study, two types of salt, such as CaCl_2 (5-100 mM) and NaCl (50-600 mM), were added at different concentrations to prepared emulsions containing 0.1-2% EWP concentrations at pH 9.0, respectively. The effects of CaCl_2 and NaCl on the particle size of emulsions were measured after 24 hours of preparation and the results are presented in Figure 5.6, respectively. In the case of emulsions with added CaCl_2 , the particle size increased as ionic strength increased from 0 to 100 mM in emulsions containing 0.1 - 0.5% EWP (Figure 5.6a). On the other hand, in 0.8 and 1.0% EWP emulsions, the particle size increased with increasing ionic strength from 0 to 10 mM but began decreasing above 10 mM, i.e. at 50 and 100 mM CaCl_2 . In case of 2% EWP emulsions, the particle size increased as the ionic strength increased from 0 to 50 mM, but decreased above 50 mM. The increase in droplet size upon CaCl_2 addition suggests droplet aggregation and flocculation (Degner et al., 2014). These droplet aggregations were believed to be caused by the Ca^{2+} ions binding to the negatively charged droplets in the emulsions resulting in the screening effect of the surface charges as well as the formation of calcium bridges between the oil droplets (Ye, Lo & Singh, 2012; Degner et al., 2014). Mine et al. (1991) reported reduced emulsifying ability and droplet aggregation on addition of 10 mM CaCl_2 to EWP-stabilised emulsions. The results obtained show the EWP-stabilised emulsions are significantly sensitive to the addition of CaCl_2 at all EWP concentrations used in this study.

The effect of NaCl concentration on the mean particle size of emulsions is shown Figure 5.6b. The results indicate that the oil droplet size increased with increasing ionic strength (0, 50, 100, 200, 400 and 600 mM) at 0.1-0.8% protein concentrations. However, at higher protein concentrations of 1 and 2%, no significant change in droplet size was observed with increasing NaCl concentration when compared to the control samples (i.e. 0 mM NaCl).

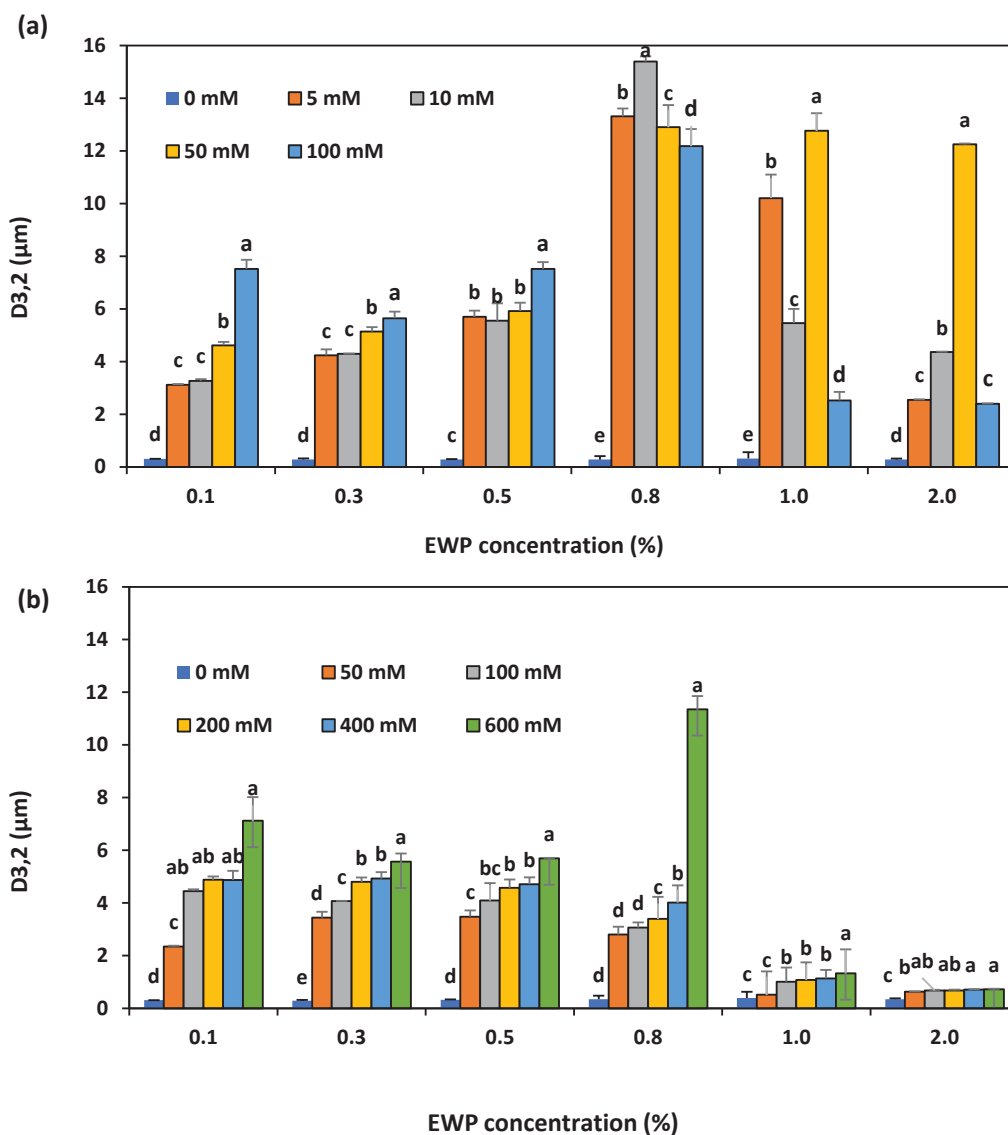


Figure 5.6: Influence of (a) CaCl₂ (0, 5, 10, 50 and 100 mM) and (b) NaCl (0, 50, 100, 200, 400 and 600 mM) on the mean particle size (D_{3,2}) of O/W emulsions prepared with 5% oil (w/w) and various EWP concentrations (0.1, 0.3, 0.5, 0.8, 1.0 and 2.0% w/w).

Chang et al. (2016) reported no significant change in droplet size in 1% EWP-stabilised emulsions at salt concentrations of 0-100 mM. At the same salt concentrations (i.e. 50 and 100 mM), when the two different sets of emulsion samples added with CaCl₂ or NaCl were compared for their oil droplet size, the oil droplets of emulsions added with CaCl₂ at the high protein concentrations (i.e. 0.8, 1.0 and 2.0%) were larger in size than the ones added with NaCl. The results obtained suggest EWP-stabilised emulsions were more sensitive to CaCl₂ than NaCl. Secondly, emulsions were more affected at high ionic strength than low

ionic strength, except for a few cases that CaCl_2 was added to the emulsions prepared with relatively high EWP concentrations of 0.8, 1.0 and 2.0%.

The stability of emulsions at low salt concentration can be due to “the salting-in effect”. At low salt concentration, the hydrated salt ions (Na^+ and Cl^-) especially the anions (Cl^-) are weakly bound to the charged group of proteins, resulting in increased solubility (Joshi et al., 2012; Yuliana, Truong, Huynh, Ho, & Ju, 2014). However, at higher salt concentrations the “salting-out effect” occurs and its effect of salt on proteins would depend on the type and ionic strength of salts (Hamada, Arakawa, Shiraki, 2009; Joshi et al., 2012). At high salt concentration, the existing available water are bound to the hydrated salt ions, thus encouraging protein-protein interactions and formation of proteins aggregates resulting in protein dehydration and reduced water capacity (Hamada et al., 2009). Furthermore, it's been suggested that at high salt concentrations, there is competition between salts and proteins to bind with water to ionize itself which reduces water availability, increase in protein dehydration and eventually resulting in the slow diffusion of protein molecules into the O/W interface. Thus, enabling protein aggregation creaming and reduction of emulsion stability (Smith & Culbertson, 2000; Joshi et al., 2012).

5.4.4.2 Emulsion stability

As shown in Figures 5.7 and 5.8, extensive aggregation and phase separation of emulsions were observed at low EWP concentration (0.1 to 0.5%), with negligible separation seen at high EWP concentration (0.8-2.0%) upon addition of CaCl_2 and NaCl . On the other hand, the control emulsion with no added NaCl and CaCl_2 was stable against aggregation, creaming and separation. The visual observation collaborated well with the particle size results obtained for emulsions with added NaCl (Figures 5.7 and 5.8). The reason for the instability at low protein concentration could be that the required protein needed to cover the emulsion oil droplets was not met and excess protein in the continuous phase was necessary for stability against flocculation induced by salt (Delahaije et al., 2013; Delahaije et al., 2015). Another possible reason for the stability at high protein concentration could be attributed to the formation of multi-layered protein shell around the oil droplets which prevented decrease in the net charge of the droplets compared to the thin layer membrane formed at low protein concentrations (Joshi et al., 2012). Furthermore, the emulsions were susceptible to separation at high ionic strength of both CaCl_2 and NaCl (Figures 5.7 and 5.8). At high ionic strength, there is an absence of strong electrostatic force among the

droplets, resulting in droplet aggregation and flocculation (Joshi et al., 2012; Yuliana, Truong, Huynh, Ho, & Ju. 2014). The results obtained indicates emulsions stabilised by EWP were more stable to salt treatment at high protein concentrations especially at 1 and 2% protein than at low protein concentrations. Furthermore, high ionic strength resulted in less emulsion stability and lastly, emulsions were more sensitive to addition of CaCl_2 than NaCl .

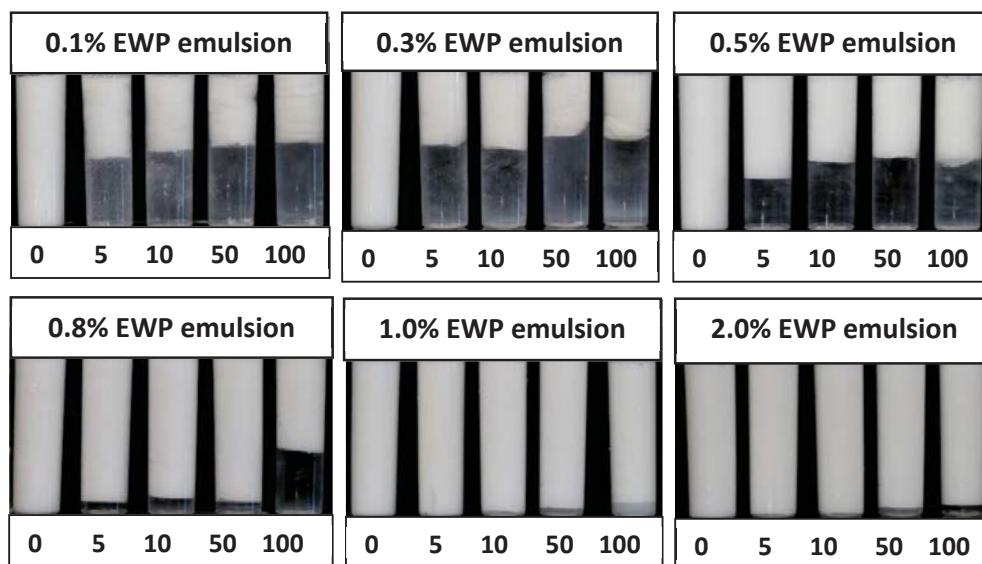


Figure 5.7: Photographs showing the influence of CaCl_2 concentration (0, 5, 10, 50 and 100 mM) on stability of emulsions containing 5% oil (w/w) and different EWP concentrations (0.1, 0.3, 0.5, 0.8, 1.0 and 2.0% w/w).

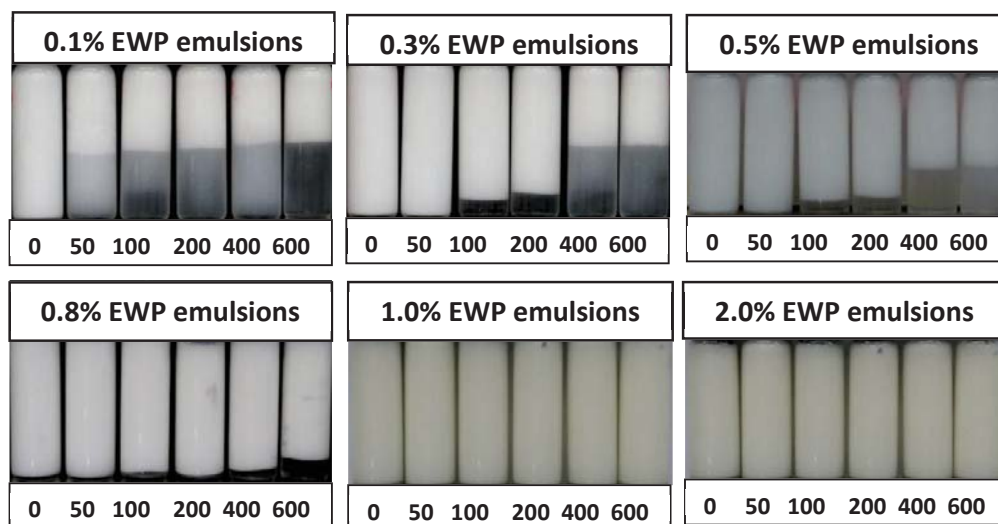


Figure 5.8: Photographs showing the influence of NaCl concentration (0, 50, 100, 200, 400 and 600mM) on emulsions containing 5% oil (w/w) and different EWP concentrations (0.1, 0.3, 0.5, 0.8, 1.0 and 2.0% w/w).

5.4.5 Effect of pH

To study the effect of pH on EWP-stabilised emulsions, the pH of EWP emulsions (8.3) prepared with 1% (w/w) EWP and 5% (w/w) oil was adjusted to pH 2, 3, 4, 5, 6, 7, 8, 9 and 10. As shown in Figure 5.9, at the pH below the pI of EWP, ζ -potential was positive and increased to be relatively high (+35 mV) at pH 2. High ζ -potential value (+73 mV) at acidic pH 3.4 was reported in acid treated EWP-stabilised emulsions (Chang et al., 2016). When the pH increased, the magnitude of the positive charge reduced, and then eventually became zero at around pH 4.5 which is the pI of ovalbumin, the predominant EWP (Alleoni, 2006) because there was a balance between the number of positively charged groups and negatively charged groups. Further increase in pH above pH 4.5, the oil droplets obtained a net negative charge, with the magnitude of droplet charge increasing as pH increased. Highest ζ -potential was obtained at pH 10 being -40 mV, indicating oil droplets were covered with proteins conferring electrostatic repulsive charge needed to maintain emulsion stability (Kulmyrzaev, Chanamai, & McClements, 2000).

The microstructure of the pH adjusted emulsions was examined as shown in Figure 5.10. The microstructure image showed no sign of droplet aggregation at acidic pH 2.0. Former investigations also revealed smaller droplets and no droplet aggregation at pH 2 and 3 in acid treated EWP emulsions (Mine et al., 1991; Chang et al., 2016). They revealed that emulsion droplets were smaller at low pH because the amount of ovalbumin adsorbed and surface hydrophobicity were maximal at acidic pH, but reduces as pH increases. Whey and soy proteins have also shown to be more stable at acidic pH than neutral pH (GoTo, Calcian, & Fink, 1990; Jiang, Chen, & Xiong, 2009). Furthermore, previous studies have revealed that globular proteins after been subjected to extensive acidic condition, undergo partial unfolding, molten globule confirmation and flexibility which helps them retain their intact structure (Raghavan & Kristinsson, 2007).

However, at pH 4 and 5 extensive droplet aggregation and flocculation was observed. Extensive droplet aggregation can be expected at these pH, which is the pI of most EWPs because there is a decrease in electrostatic repulsion between the highly-charged droplets causing aggregation of proteins and depletion flocculation (Ray & Rousseau, 2013; Niu et al., 2015). Chang et al. (2016) reported large droplet aggregates at pH 4.2 in a study of physicochemical and stability of acid and acid-treated EWP at various pH (3.0, 3.4, 3.8 and 4.2). Nevertheless, at control pH (8.3) the emulsions had uniformly distributed oil droplets

with no sign of droplet aggregation. No sign of droplet aggregation was also observed at neutral pH 9 and 10. This could be attributed to the overall net negative charge on the proteins, which contributed to strong electrostatic repulsion force that prevented the emulsions from been destabilised by aggregation or flocculation (Yuliana et al., 2014).

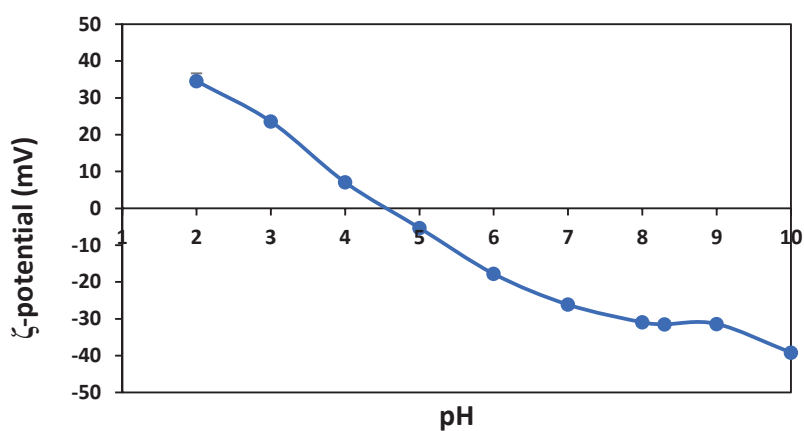


Figure 5.9: Influence of pH on ζ -potential of emulsion prepared with 1% (w/w) EWP and 5% (w/w) oil. Data represents mean $\pm$ standard deviation of two independent measurements with duplicates (n=6).

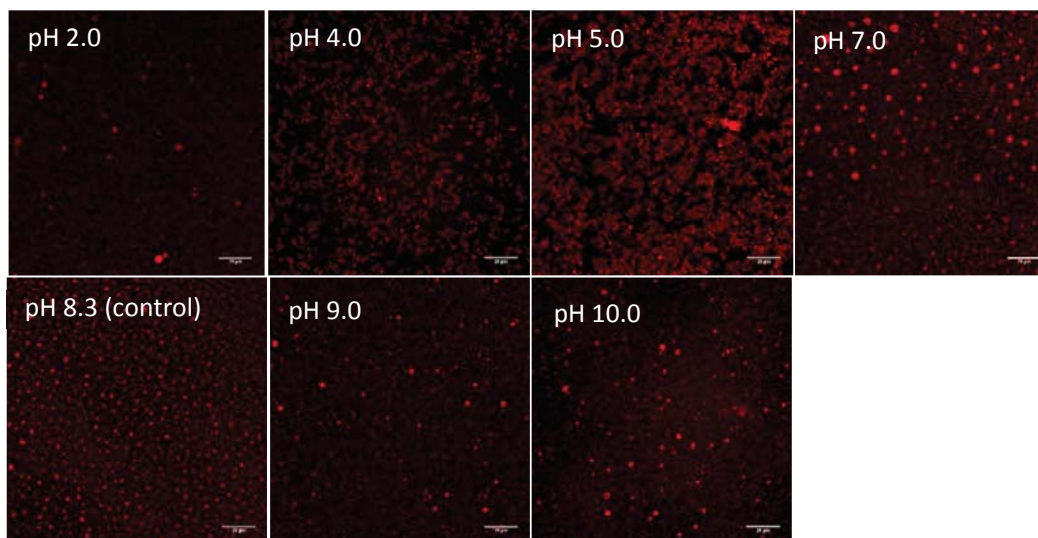


Figure 5.10: Confocal images of pH adjusted (2, 4, 5, 7, 8.3, 9 and 10) of emulsions prepared with 1% (w/w) EWP and 5% (w/w) oil. The scale bar inserted represents 20 μ m.

5.5 Conclusions

This experimental work compared the formation and properties of 5% (w/w) O/W emulsions stabilised by different concentration of EWP (0.1-2% w/w) prepared at pH 8.3. Furthermore, the behaviour of these emulsions under different conditions (heat treatment, freezing and thawing, addition of salt (NaCl and CaCl₂) and pH) was observed. Emulsions prepared with 0.1-1% EWP concentration generated little visible aggregates. At 2% aggregates formed was significantly more than those obtained at lower EWP concentrations (0.1-1%). No sign of instability (aggregation/flocculation) was observed in the prepared emulsions even after 5 days. The droplet size of the emulsion increased with increasing protein concentrations in which highest droplet size was achieved (703.7 nm) at 2% and lowest at 0.3% (242.1 nm). The large droplet size observe at 2% protein concentration could be attributed to flocculation by depletion arising from the presence of excess protein in the continuous phase. On the other hand, the small droplet size observed at 0.3%, indicates that the protein concentration was sufficient to fully cover the newly formed emulsion droplet, hence forming sufficient thick layer around the droplets and providing stability against aggregation and flocculation. ζ -potential of the emulsion droplet ranged from -35.3 to -39.2 mV and no significant effect of protein concentration was observed.

When compared to control emulsions (with no heat treatment), heat treatments at temperatures 60, 65, 70, 80 and 90°C and holding time 0 and 30 minutes was found not to have any significant effect on droplet size of emulsion containing different protein concentrations. It was also found that freezing at -20°C and thawing 4°C and 20°C respectively resulted in phase separation irrespective of their protein concentration. The emulsions were separated into a rich opaque droplet layer floating at the top and a transparent or slightly turbid aqueous layer at the bottom. The emulsions were highly susceptible to extensive flocculation at low protein concentrations (0.1- 0.8%) on addition of 5-100 mM CaCl₂ and 50-600 mM NaCl. Addition of excess protein (1-2%) significantly improved the stability of the emulsions against salt-induced flocculation. At high protein concentration, multi-layered protective covering is generated around the emulsion droplets, thus providing stability against flocculation. Furthermore, the emulsions were more sensitive to the effect of CaCl₂ than NaCl. At acidic pH (2.0), emulsions were found to be stable against droplet aggregation. However, at pH 4-5 close to the isoelectric point (pI) of EWP, extensive droplet aggregation was observed to occur as expected due to loss of electrostatic repulsive forces between oil droplets. At alkaline pH (pH 9 and 10), stable

emulsions were also produced. It's been reported that at acidic pH or alkaline pH, the proteins have either an overall net positive charge or negative charge which encourages strong electrostatic repulsion among the droplets, hence stability against aggregation, coalescence and flocculation (Yuliana et al., 2014). This study highlights the importance of protein concentration in the formulation and production of stable egg white emulsions.

Chapter 6. Emulsifying properties of EWP hydrolysates

6.1 Abstract

In this study, degree of hydrolysis (DH) and emulsifying properties of egg white protein hydrolysates were investigated. Hydrolysis was carried out separately with three proteases (bromelain, ficin and papain) to determine the effects of enzyme concentration (0, 0.3, 0.5, 1, 2 and 4% w/w) and hydrolysis times (0, 30, 60 and 120 minutes) on the DH and peptide molecular weight distribution. DH was observed to increase significantly ($p < 0.05$) with increasing enzyme concentration and hydrolysis time. A significant difference ($p < 0.05$) among the enzymes was only observed with 4% E/S ratio at 120 minutes hydrolysis, with papain yielding the highest DH 7.69% while bromelain and ficin yielded similar DH levels of 5.03% and 4.99%, respectively. The results of SDS-PAGE analysis showed that major EWP proteins, such as ovotransferrin and ovalbumin, were completely digested into smaller peptides at all enzyme concentrations and hydrolysis times used. Furthermore, the effects of enzyme concentration, DH and hydrolysis time on the emulsifying properties of hydrolysed EWP prepared with bromelain and ficin were also investigated. Enzymatic hydrolysis significantly improved the appearance of EWP-stabilised emulsions by producing a clear emulsion free from aggregates compared to emulsions prepared with original EWP with lots of large aggregates formed in the emulsions. When compared with original EWP, emulsions containing 10% w/w oil and various EWP concentrations (1, 5 and 10% w/w) prepared with hydrolysed EWP (4% E/S, DH 5.16%) yielded smaller droplet size (0.66-0.98 μm) than those of control emulsions (1.22-39.35 μm). However, the hydrolysed emulsions separated immediately after homogenisation at all protein concentrations used while for the control emulsions, phase separation occurred in only emulsions stabilised with 5 and 10% (w/w) EWP. Gelation at 5 and 10% (w/w) EWP concentration and aggregation and phase separation at 1% (w/w) were seen after heat treatment. Additionally, emulsions prepared with 1% E/S ficin (DH 4.03% and 4.96%) and 0.3% E/S (DH 3.01%) yielded smaller droplets size (0.75-1.27 μm) than droplet size (6.40-7.37 μm) of emulsions prepared with bromelain (1% E/S, DH 4.10% and 4.87%). Droplet size decreased as hydrolysis time increased from 2 to 4 hours for both ficin and bromelain EWP hydrolysates with phase separation occurring the following day after preparation. Thus, DH and enzyme type were found to have an influence on the emulsifying properties of EWP hydrolysates.

6.2 Introduction

Enzymatic hydrolysis is another efficient method of expanding the added value of food proteins by modifying their physical, nutritional and functional properties without losing its nutritional value (Pacheco-Aguilar, Mazorra-Manzano, & Ramírez-Suarez, 2008). Enzymatic treatments modify the properties of food proteins by reducing their molecular weight, increasing the number of polar and ionisable groups and exposing hidden hydrophobic groups from the protein interior (Klompong, Benjakul, Kantachote, & Shahidi, 2007; Foegeding & Davis, 2011). It also increases the solubility of food proteins over a wide range of pH, which is important in stabilisation of foams and emulsions (Gbogouri, Linder, Fanni, & Parmentier, 2004; Liu, Kong, Xiong, & Xia, 2010).

Proteolytic enzymes are commonly used for enzymatic hydrolysis of food proteins and can be obtained from plants (e.g. bromelain, ficin and papain), animals (e.g. trypsin, chymotrypsin, pepsin and pancreatin) or microbial sources (e.g. alcalase, protamex, flavozyme, neutrase and subtilisin) (Guerard, Dufosse, La Broise, & Binet, 2001). These enzymes can be used singly, in combination or sequentially to produce protein hydrolysates. Different enzymes vary in their mode of reaction or type of hydrolysate they produce due to their ability to bind to peptide bonds at specific active sites (Herpandi, Rosma, & Nadiyah, 2011). The narrower the enzyme specificity, the larger the number of peptides; while the wider the specificity, the smaller the number of peptides produced (Kristinsson & Rasco, 2000). The hydrolysis conditions, such as pH, ionic strength, temperature and enzyme/substrate ratio (E/S), for preparing protein hydrolysates are selected within the specific activity range of the enzymes and the required degree of hydrolysis (DH) (Alder-Nissen, 1993; El-Salam & El-Shibiny, 2017).

Protein hydrolysates are a mixture of dipeptides, tripeptides, oligopeptides, polypeptides and free amino acids and are classified based on their DH into partial hydrolysates and extensive hydrolysates (Kristinsson & Rasco, 2000; Schaafmsa, 2009; El-Salam & El-Shibiny, 2017). Partially hydrolysed proteins are used for improving a protein's functional property (Kristinsson & Rasco, 2000). In the food industry, they are used as protein supplements, milk replacers, flavour enhancers in confectionary products, beverage stabilisers and surimi production (Kristinsson & Rasco, 2000; El-Salam & El-Shibiny, 2017). On the other hand, extensive hydrolysed proteins are used mainly in clinical and normal nutrition (El-Salam & El-Shibiny, 2017). In addition to functional properties,

protein hydrolysates have been reported to possess antioxidant properties, thus ingesting antioxidant proteins could reduce oxidative damage in the human body (Sakanaka et al., 2004; Noh & Suh, 2015; Chen, Chang, Wang, & Chen, 2009).

Controlled enzymatic hydrolysis can improve the emulsifying properties of proteins as extensive hydrolysis results in loss of emulsifying properties (Klompong, Benjakul, Kantachote, & Shahidi, 2007; Horax, Vallecios, Hettiarachchy, Osorio, & Pengyin, 2017). The DH and molecular weight distribution (MWD) are important hydrolysates characteristics that can affect emulsifying properties of proteins. The DH measures a protein's hydrolytic degradation and is used as an indicator for comparing different proteolytic process (Cho et al., 2014; Noh & Suh, 2015). It also influences the amino acid composition and size of peptides. Studies have shown that stable emulsions are generally produced at low DH (Shahidi et al., 1995; van der Ven et al., 2001; Klompong, Benjakul, Kantachote, & Shahidi, 2007). van der Ven et al. (2001) reported destabilisation of whey protein emulsions at DH >8%. Greater emulsion stability was also observed when DH was low ($\leq 5\%$) for yellow stripe trevally hydrolysate (Klompong et al., 2007). With regards to the dimension of molecular weights, a minimum peptide length is required for good emulsifying properties of protein hydrolysates (van der Ven, Gruppen, de Bont, & Voragen, 2001; Chen, Chi, Zhao & Xu, 2012). Singh & Dalgleish (1998) found that a peptide length of 500 Da only was needed to stabilise whey protein emulsions, while van der Ven et al. (2001) revealed that high molecular weight peptides >3 kDa were required for emulsion stability of casein and whey hydrolysates.

Egg white proteins are key ingredients in food products (meringues, ice creams, meat products and baked products) due to their multi-functional properties such as gelling, foaming, heat setting, binding and emulsifying properties (Mine, 1995; Erçelebi & Ibanoglu, 2009). Furthermore, hydrolysates produced from EWP have been reported to be highly nutritious, more digestible and water soluble when compared to original EWP because of their low molecular weights (Yujie, Bo, Bo, Mingruo, 2006; Chen et al., 2012). Thus far, most studies have focused on the antioxidant and angiotensin converting enzyme (ACE) inhibition properties of EWP hydrolysates. However, information regarding the functional properties of EWP hydrolysates as affected by DH and enzyme type is still limited. Therefore, the objectives of this study were to investigate the effects of enzyme type, enzyme concentration and hydrolysis time on the DH of EWP prepared with

bromelain, ficin and papain. In addition, the effects of DH, hydrolysis time, enzyme type (bromelain and ficin) on the emulsifying properties of EWP were evaluated. As previously reported in Chapter 4, EWP emulsions with less visible aggregates were only achieved in emulsions prepared with 1% EWP/5% oil and 3% EWP/1% oil. Above the stated protein or oil concentrations, large visible aggregates were produced. Thus, this study also aimed at investigating the efficiency of enzymatic hydrolysis in reducing the formation aggregates in emulsions prepared with high concentration of EWP (e.g. 5 and 10%) and high oil concentration (10% w/w).

6.3 Materials and Methods

6.3.1 Materials

Pasteurised egg white liquid (EWL) containing 10% w/w protein (Zeagold Foods, New Zealand) and canola oil were purchased from a local supermarket. Enzymes bromelain (lyophilized powder, ≥ 3 units/mg protein), ficin (powder, ≥ 0.1 units/mg solid) and papain (≥ 10 units/mg lyophilized powder) were obtained from Sigma-Aldrich Inc. Table 6.1 summarises the characteristics of each enzyme used. For the preparation of the ortho-phthalaldehyde (OPA) reagent, OPA powder (97% HPLC grade) and sodium tetraborate (98% anhydrous) were purchased from Sigma-Aldrich Inc. (Australia), while sodium dodecyl sulphate (SDS) (99%), 2-mercaptoethanol (98%), methanol (high grade anhydrous alcohol) were purchased from Thermo Fisher Scientific Inc. (New Zealand). For sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), 10x Tris/Tricine/SDS Running Buffer (#1610744), 2x Laemmli sample buffer (#1610737), 4-15% Mini-PROTEAN[®] TGX[™] precast gels, and molecular weight standards (Precision Plus Protein Unstained Standards Catalog #161-0317) were purchased from Bio-Rad Laboratories (Hercules, CA, USA). The molecular weight standards comprised of a mixture of 10 proteins in the molecular range of 10 to 250 kDa (10, 15, 20, 25, 37, 50, 75, 100, 150 and 250 kDa). Coomassie Brilliant Blue (Catalog #1610406) was purchased from Bio-Rad Laboratories (Hercules, CA, USA) and was used for preparing the staining solution. Glacial acetic acid (100%) was purchased from BDH Chemicals (Poole, England). Glacial acetic and methanol were used in preparing staining and de-staining solutions.

Table 6.1: Enzyme characteristics

Enzyme	Source	Type	Molecular weight (kDa)	Optimum conditions	
				Temperature (°C)	pH
Bromelain	Pineapple stem	Thiol protease	26-37	40-65	4.0-9.0
Ficin	Fig tree	Thiol protease	25-26	45-55	5.0-6.0
Papain	Papaya latex	Thiol protease	20-24	60-70	6.0-7.0

Source: Noh & Suh (2015) and Manzoor, Nawaz, Mukhtar & Haq (2016)

6.3.2 Preparation of EWP hydrolysates

In this study, four categories of egg white protein hydrolysates (EWP) were prepared. Firstly, to investigate the effects of enzyme type, enzyme concentration and hydrolysis time on the DH of EWP, EWP was prepared with bromelain, ficin and papain using different E/S ratios (0.3, 0.5, 1, 2 and 4% w/w) at different reaction times (0, 30, 60 or 120 minutes). Secondly, to investigate the effect of high DH on the emulsifying properties of EWP, EWP solution was hydrolysed with bromelain at a high E/S ratio of 4% (w/w) for 2 hours (enzyme bromelain chosen due to its low cost and appreciable DH observed from the results of preliminary experiments of this study). Thirdly, to investigate the effects of enzyme type and hydrolysis time on emulsifying properties of EWP, EWP solution was hydrolysed separately with bromelain and ficin at an E/S ratio of 1% (w/w) for 2 and 4 hours. Lastly, to investigate the emulsifying properties of EWP prepared ficin with low DH, EWP solution was hydrolysed with ficin at an E/S ratio of 0.3% (w/w) for 4 hours.

6.3.2.1 Effects of enzyme type, enzyme concentration and hydrolysis time on DH of EWP

The hydrolysis of EWP solution was carried out according to the method of Chang et al. (2017) with slight modification. EWL containing 10% (w/w) protein was stirred for 30 minutes to ensure uniform consistency, after which it was adjusted to pH 6.0 using 1 M HCl and incubated at 50°C for 20 minutes before enzyme addition. Enzymes (bromelain, ficin and papain) were added at an E/S ratio of 0.3, 0.5, 1, 2 and 4% (accounted for the weight ratio of the total protein). Hydrolysis was carried out for 120 minutes at 50°C under constant stirring. The pH was maintained during hydrolysis for optimal value with 1 M HCl. EWP samples were withdrawn at different time intervals of 0, 30, 60 and 120 minutes followed by heat treatment at 85°C for 3 minutes and then freezing immediately to

stop enzymatic reactions. Control EWL was treated by titrating to pH 6 with 1 M NaOH and then heated at 50°C for 2 hours. The hydrolysis temperature and pH chosen were within the optimum range of enzyme activity as specified by the manufacturers and some previous studies reported in the literature (Cho et al., 2014; Noh & Suh, 2015).

6.3.2.2 Effect of hydrolysis on emulsifying property of EWP

To investigate the effect of high DH on the emulsifying properties of EWP, EWP solution was hydrolysed with bromelain at a high E/S ratio of 4% (w/w) at pH 6.0, 50°C for 2 hours. The hydrolysed EWP was used immediately for emulsion preparation without further treatment. Hydrolysis conditions were the same as described above.

To investigate the effects of enzyme type and hydrolysis time on emulsifying properties of EWP, EWP solution was hydrolysed separately with bromelain and ficin at an E/S ratio of 1% (w/w) for 2 and 4 hours. Hydrolysis conditions are the same as described above.

Lastly, to investigate the emulsifying properties of EWP prepared with ficin at low DH, EWP solution was hydrolysed with ficin at an E/S ratio of 0.3% (w/w) for 4 hours. Hydrolysis conditions are the same as described above. The hydrolysis conditions for preparing EWPHs are shown in Table 6.2.

Table 6.2: Hydrolysis conditions for preparing EWPH

Enzyme	pH	Temperature (°C)	E/S ratio (%)	Hydrolysis time (hours)
Bromelain	6.0	50	4	2
Bromelain	6.0	50	1	2
Bromelain	6.0	50	1	4
Ficin	6.0	50	1	2
Ficin	6.0	50	1	4
Ficin	6.0	50	0.3	4

6.3.3 DH

The ortho-phthalaldehyde (OPA) method was used to measure the DH of the hydrolyzed samples following the method of Church, Swaisgood, Porter & Catignani (1983) and Spellman, McEvoy, O’Cuinn & FitzGerald (2003). The OPA reagent was prepared as follows; 160 mg OPA powder (dissolved in 4 ml methanol) was combined with 100 ml sodium tetraborate and 10 ml of sodium dodecyl sulfate (SDS), in concentration of 0.1

mol/L and 20%, respectively. After which 400 µl mercaptoethanol was added and the final mixture was fixed to 200 ml with distilled water. The OPA reagent was covered with aluminium foil to protect it from light and stirred for at least one hour before use. The samples were diluted with distilled water to a concentration of 5 mg/ml. The OPA assay was carried out by adding 200 µl of sample to 4 ml OPA reagent and allowed to stand for 2 minutes before measuring the absorbance at 340 nm wavelength. The DH was calculated using the following formula;

$$DH (\%) = 100 \left(\frac{(\Delta Abs * M * d) / N}{\epsilon * c} \right)$$

$$DH (\%) = 100 \left(\frac{(\Delta Abs 45000 d) / 385}{6000 c} \right)$$

Therefore;

$$DH (\%) = \frac{\Delta Abs 1.948 d}{c}$$

where ΔAbs = absorbance difference between original and hydrolysed samples; M = average molecular weight of EWP (45000 Da); d = dilution factor (20); ϵ = extinction coefficient at 340 nm ($6000 \text{ mol}^{-1} \text{ cm}^{-1}$); c = protein concentration (5 mg/ml); N = average number of peptide bonds per protein molecule (385).

6.3.4 SDS-PAGE analysis

The EWPH samples were diluted with distilled water to 1:100 ratio, then 100 µl of sample buffer was added to the mixtures and heated for 5 minutes at 95°C. 10 µl of each sample and 10 µl of the molecular weight standard were loaded into the wells of the precast acrylamide gels (4% stacking gel and 15% separating gel). Electrophoresis was carried using a Mini-PROTEAN® tetra cell (Bio-Rad Laboratories, Richmond, CA, USA) at 40 mA for 45 minutes. The gels were removed carefully and immediately transferred into a plastic container for staining. Staining was carried out overnight with 0.3% (w/v) Coomassie Brilliant Blue solution with 50% methanol and 10% glacial acetic acid and then de-stained with a solution containing 40% methanol and 10% glacial acetic acid. The de-staining solution was changed 3 to 4 times until the gel background was clear. Images of the gels were obtained using a ChemiDoc XRS+ (Bio-Rad Laboratories, Richmond, CA, USA) scanning densitometer software.

6.3.5 Preparation of emulsions

To evaluate the emulsifying properties of EWPH, three categories of emulsions were prepared according to the type of EWPH as previously described in Section 6.3.2.2. Firstly, to compare the emulsifying ability of original egg white protein (OEWPH) and EWPH hydrolysed with 4% bromelain, the protein samples were diluted to a protein concentration of 1% and 5% (w/w) with distilled water. Then, the protein solutions (containing 1, 5 and 10% EWP concentrations) were mixed with canola oil at a weight ratio of 10:90 and pre-homogenised using an Ultra-Turrax blender (IKA T25, Staufen, Germany) at 13,000 rpm for 2 minutes to obtain coarse emulsions. Fine emulsions were prepared by passing the coarse emulsions 4 times through a 2-stage high pressure homogeniser (APV 2000, APV Manufacturing, Poland) at 500/50 MPa. The emulsions prepared were stored at 4°C until further analysis. For the second set of emulsions, EWPH prepared separately with bromelain and ficin at an E/S ratio of 1% (w/w) for 2 and 4 hours were diluted to a protein concentration of 1% (w/w). Canola oil was mixed with each protein solution at a ratio of 10:90 and then homogenised to obtain fine emulsions as described above. Lastly, EWPH prepared with 0.3% ficin hydrolysed for 4 hours was diluted to a protein concentration of 1% (w/w) and fine emulsions was prepared same as the other samples as described above. All emulsions were prepared in at least duplicates. The formulations for all the emulsions are shown below in Table 6.3.

Table 6.3: Formulations of emulsions with different types of EWPH

Experiment	OEWP/EWPH concentration (% w/w)	Oil to aqueous phase ratio	EWL (g)	Oil (g)	Total emulsion (g)
OEWP	1	10:90	270	30	300
	5	10:90	270	30	300
	10	10:90	270	30	300
4% B	1	10:90	270	30	300
	5	10:90	270	30	300
	10	10:90	270	30	300
1% F-2 hours	1	10:90	270	30	300
1% F-4 hours	1	10:90	270	30	300
1% B-2 hours	1	10:90	270	30	300
1% B-4 hours	1	10:90	270	30	300
0.3% F-4 hours	1	10:90	270	30	300

OEWP indicates emulsions produced from original egg white protein; 4% B: represent emulsions produced with 4% bromelain EWPH; 1% F-2 hours: emulsions prepared with 1% ficin obtained after 2 hours; 1% F-4 hours: with 1% ficin obtained after 4 hours; 1% B-2 hours: with 1% bromelain obtained after 2 hours; 1% B-4 hours: with 1% bromelain obtained after 4 hours; 0.3% F-4 hours: with 0.3% ficin obtained after 4 hours.

6.3.6 Particle size distribution

Laser light scattering method was used to measure the particle size and size distribution of emulsion droplets using a Malvern Mastersizer 2000 (Malvern Instruments Ltd, Worcestershire, UK). About 1 to 5 ml of emulsion was injected into a sample dispersing chamber (containing distilled water) equipped with a flow pump connected to an optical chamber for the measurement of emulsion droplet size. The relative refractive index of water (dispersant) was set to 1.333, while 1.465 was used as the relative refractive index of canola oil. At least three measurements of each emulsion sample were made from two independent samples. The mean diameter of droplet size was reported as Sauter mean diameter ($D_{3,2}$) and span.

6.3.7 Effect of thermal treatment

To determine the effect of thermal treatment on stability of the hydrolysed emulsions, the emulsion sample prepared with 4% bromelain EWPH (containing 10% (w/w) protein concentration) was diluted to a protein concentration of 1% and 5%. Then 10 ml of the EWPH emulsion containing 1, 5 and 10% protein were placed in glass test tubes and incubated in a water bath set at different temperatures (60, 65, 70, 80 and 90°C) for 0 and 30 minutes and then quickly placed in an ice water bath to cool to 20°C. Particle size measurement of the emulsions was carried out the following day using a Malvern 2000 Mastersizer as previously described in Section 6.3.6.

6.3.8 Zeta potential (ζ -potential) measurements

ζ -potential measurement of the emulsions was carried using a Malvern Zetasizer Nano ZS series (Malvern Instruments Ltd, Worcestershire, UK). Emulsion samples were diluted with distilled water at a ratio of 1:100 (v/v) and placed in a disposable ζ -potential cell (“Size and Zeta” folded capillary cell, model DTS1070). Sample measurements was carried out after 60 seconds equilibration at 25°C inside the instrument. ζ -potential was calculated using the Smoluchowsky mathematical model by the instrument software (Zetasizer Software, Version 7.10, Malvern Instruments Ltd, Worcestershire, UK).

6.3.9 Microscopic examinations

The microstructure of the emulsions was examined using an Axiolab A reflected light microscope (Zeiss, Berlin, Germany). Emulsion samples were diluted at a ratio of 1:10 (v/v) with distilled water. About 30 μ l of the diluted emulsion was placed in a microscope slide and covered with a coverslip which was viewed with 40 x objective lens. Microscopic images were chosen from at least five similar images.

6.3.10 Colour measurement

The colours of the emulsions were measured using a Minolta Colorimeter (Chroma Meter CR300, Minolta Camera Co., Japan). The colorimeter was calibrated with a white standard tile (Standard NO.22933022, Minolta Co. Ltd., Japan) with values: $Y=92.40$, $x=0.3138$, $y=0.3192$. The standard illuminant “C” was used as the light source. The CIE L , a , b values were used in this study, where L indicates lightness of the sample (0 = black and 100 = diffuse white); a indicates coordinate positions between red and green (+ve values represent red and -ve values represent green); b indicates coordinate positions between blue and yellow (+ve values represent yellow and -ve values represent blue). Measurement of each

emulsion sample was done in triplicates. Colour measurements were carried out the following day after emulsion preparation.

6.3.11 Data analysis

All experiments and sample analyses were carried out in duplicate. Results are presented as mean and standard deviation. Data were analysed statistically by analysis of variance (ANOVA) using Minitab statistical software version 17 (Minitab Inc., USA). Significant difference between means was analysed by Turkey's HSD method at a confidence level of 95% ($\alpha = 0.05$).

6.4 Results and Discussion

6.4.1 Effect of enzyme concentration and hydrolysis time on DH of EWP

The objective of this study was to investigate the effect of enzyme concentration and hydrolysis time on DH of EWP hydrolysates prepared with three enzymes bromelain, ficin and papain. In this study, five E/S ratios (0.3, 0.5, 1, 2 and 4%) and four hydrolysis time (0, 30, 60, 120 minutes) were used for each enzyme and their DH monitored using the OPA method. The DH measures the extent of enzymatic breakdown of proteins and is a widely used parameter for comparing different proteolytic process. Table 6.4 shows DH results obtained for the three enzymes at different enzyme concentrations (0.3, 0.5, 1, 2 and 4%) and hydrolysis times (0, 30, 60, 120).

As can be seen in Table 6.4, with increasing hydrolysis time from 0 to 120 minutes, there was a gradual increasing trend in DH values at all enzyme concentrations used for the bromelain, ficin and papain. Highest DH values were obtained at 120 minutes while lowest DH values were obtained at 0 minutes. Treatments at 30 and 60 minutes resulted in similar DH values in all E/S used. The results also revealed increasing DH values with increase in enzyme concentration for the three enzymes type. For bromelain, treatment with 4% E/S ratio resulted in highest DH values of 2.28%, 3.63%, 4.31% and 5.03% at 0, 30, 60 and 120 minutes respectively; 2.44%, 3.60%, 3.88% and 5.56% for ficin treatments and 2.70%, 4.47%, 4.98% and 7.69% for papain treatments. At each hydrolysis time, the DH values of 4% E/S ratio was higher ($p < 0.05$) than those of other E/S ratios (0.3, 0.5, 1 and 2%). The results suggest that more peptide bonds were likely to be cleaved in the presence of a large amount of added enzymes (Klompong et al., 2007).

Table 6.4: Degree of hydrolysis (%) of EWP hydrolysates hydrolysed with three different enzymes using various E/S ratios (0.3, 0.5, 1, 2 and 4%). Hydrolysis was carried out at 50°C for 120 minutes with samples taken out at 0, 30, 60 and 120 minutes.

Enzyme concentration (%)	Enzyme type	Time (minutes)			
		0	30	60	120
0.3	Bromelain	0.50 ± 0.15 ^{Ac}	1.16 ± 0.26 ^{Ab}	1.52 ± 0.26 ^{Ab}	2.33 ± 0.31 ^{Aa}
	Ficin	0.57 ± 0.15 ^{Ac}	1.30 ± 0.06 ^{Ab}	1.44 ± 0.05 ^{Ab}	2.35 ± 0.05 ^{Aa}
	Papain	0.58 ± 0.08 ^{Ad}	1.34 ± 0.21 ^{Ac}	1.54 ± 0.06 ^{Ab}	2.01 ± 0.04 ^{Aa}
0.5	Bromelain	0.99 ± 0.20 ^{Ad}	1.54 ± 0.45 ^{Ac}	1.93 ± 0.26 ^{Ab}	2.74 ± 0.25 ^{Aa}
	Ficin	1.03 ± 0.04 ^{Ac}	2.07 ± 0.20 ^{Ab}	2.37 ± 0.40 ^{Aab}	2.91 ± 0.07 ^{Aa}
	Papain	0.90 ± 0.28 ^{Ad}	1.68 ± 0.04 ^{Ac}	2.00 ± 0.17 ^{Ab}	2.49 ± 0.14 ^{Aa}
1	Bromelain	1.38 ± 0.07 ^{Ac}	2.18 ± 0.01 ^{Bc}	2.63 ± 0.17 ^{Ab}	3.30 ± 0.09 ^{Aa}
	Ficin	1.39 ± 0.08 ^{Ac}	2.57 ± 0.07 ^{Ab}	2.78 ± 0.02 ^{Ab}	3.34 ± 0.03 ^{Aa}
	Papain	1.22 ± 0.35 ^{Ad}	2.02 ± 0.01 ^{Bc}	2.44 ± 0.46 ^{Ab}	3.42 ± 0.10 ^{Aa}
2	Bromelain	1.94 ± 0.04 ^{Ad}	3.08 ± 0.23 ^{Ac}	3.48 ± 0.14 ^{Ab}	4.07 ± 0.23 ^{Ba}
	Ficin	1.86 ± 0.08 ^{Ad}	3.01 ± 0.07 ^{Ac}	3.47 ± 0.16 ^{Ab}	4.15 ± 0.08 ^{Ba}
	Papain	2.04 ± 0.40 ^{Ac}	2.77 ± 0.50 ^{Ab}	3.48 ± 0.27 ^{Ab}	4.58 ± 0.27 ^{Aa}
4	Bromelain	2.28 ± 0.07 ^{Bd}	3.64 ± 0.20 ^{Ac}	4.31 ± 0.03 ^{Bb}	5.03 ± 0.26 ^{Ba}
	Ficin	2.44 ± 0.54 ^{Bc}	3.60 ± 0.20 ^{Ab}	3.88 ± 0.16 ^{Bb}	5.56 ± 0.43 ^{Ba}
	Papain	2.70 ± 0.33 ^{Ad}	4.47 ± 0.56 ^{Ac}	4.98 ± 0.45 ^{Ab}	7.69 ± 0.33 ^{Aa}

^{a-d} Means sharing different lowercase letters in the same row are significantly different ($p < 0.05$). ^{A-C} Means sharing different uppercase letters in the same column are significantly different ($p < 0.05$). Results are presented as the means ± SD for n=6.

The increase in DH value with increase in enzyme concentration and hydrolysis time was similar to the results reported for EWP hydrolysates (Chen et al., 2012; Noh & Suh, 2015; Chang et al., 2017); yellow stripe trevally hydrolysate (Klompong et al., 2007); salmon hydrolysates (Kristinsson & Rasco, 2000; Gbogouri et al., 2004); Atlantic cod hydrolysates (Aspmo, Horn, & Eijsink, 2005). Noh & Suh. (2015) reported that EWP hydrolysate prepared with alcalase showed an increase in DH as E/S ratio increased from 2 to 5%. They also reported a sharp increase in DH in the first 8 hours of hydrolysis. Increase in DH as a result of increasing enzyme concentration (1 to 5% w/w) and hydrolysis time (1 to 6 hours) was also reported in fish hydrolysates prepared with flavourzyme and kojizyme (Nilsang, Lertsiri, Suphantharika, & Assavanig, 2005). However, since enzymes are the most

expensive factor in any hydrolytic process, minimising the quantity of enzymes used is very important (Aspmo et al., 2005). Manufacturers advise an E/S ratio between 2 and 10:100 for complete digestion of proteins (Gibbs, Zougman, Masse, & Mulligan, 2004). Additionally, most researchers recommend a range of 1 to 3% as an enzyme economic usage range which can boost up to 2.4% DH (Wu, Wang, & Xu., 2008). To summarise, DH of EWPH prepared with bromelain, ficin and papain increased with increasing enzyme concentration and hydrolysis time. High DH of 5.03%, 4.99% and 7.06% was obtained at 4% enzyme concentration for bromelain, ficin and papain, respectively, after 120 minutes hydrolysis.

6.4.2 Effect of enzyme type on DH of EWPH

Studies on comparison of enzymes are complicated because of their different specificities, cleavage sites and optimal working conditions (Aspmo et al., 2005; Nimalaratne, Bandara, & Wu, 2005). The DH of EWP hydrolysates prepared with bromelain, ficin and papain at various E/S ratios (0.3, 0.5, 1, 2 and 4% w/w) for 120 minutes was compared. As shown in Table 6.4, no significant difference ($p < 0.05$) in DH levels between the samples treated with bromelain, ficin and papain was observed at 0.3 to 2% E/S ratios. However, significant difference ($p < 0.05$) among the enzymes was significantly observed at 4% enzyme concentration with papain EWPH having the highest DH (7.69%). Furthermore, bromelain and ficin showed similar DH levels of 5.03% and 4.99%, respectively, while the control with no enzymatic hydrolysis had a DH of 0%. Noh & Suh (2015) reported EWPH prepared with alcalase had a higher DH (43.2%) than hydrolysates prepared with other enzymes, such as neutrase, protamex, flavouzyme, collupulin and ficin. In another study, Cho et al. (2014) hydrolysed dried EWP with the same enzymes and conditions used by Noh & Suh (2015) but reported neutrase yielded the highest DH (23.4%).

Although DH is affected by many factors (e.g. temperature, pH, nature of substrate, substrate/water ratio or enzyme/substrate ratio), enzyme type remarkably affects the DH and properties of the final hydrolysate (El-Salam & El-Shibiny, 2017). This is because the variety of peptides and amino acids generated during hydrolysis depend on specificity and cleavage sites of each enzyme (Wu et al., 2003). Papain is a cysteine protease produced from *Carica papaya*. The enzyme has been reported very useful in tenderizing meat proteins by cleaving the peptide bonds of basic amino acids. On the other hand, bromelain and ficin, also cysteine proteases have preferential cleavage of peptide bonds at the basic

amino acid or aromatic amino acids groups (El-Salam & El-Shibiny, 2017). In summary, the results revealed no difference in the DH of hydrolysates prepared with bromelain, ficin and papain yielded higher DH at 4% enzyme concentration than that of bromelain and ficin. In conclusion, with enzyme concentrations 0.3 to 2%, the hydrolysates prepared with bromelain, ficin and papain showed similar DH levels. However, at 4% papain yielded higher DH than bromelain and ficin.

6.4.3 SDS-PAGE analysis

To investigate the main components of EWP hydrolysed using various enzymes (bromelain, ficin and papain), various enzyme concentrations (0.5, 1, 2 and 4% w/w) and hydrolysis time (0, 30, 60 and 120 minutes), reducing SDS-PAGE analysis was conducted. As shown in Figure 6.1a, 6.1b and 6.1c original EWP (control) comprised mainly of ovotransferrin, ovalbumin and lysozyme with molecular weight (MW) of 77.7 kDa, 44.5 kDa and 14.4 kDa, respectively. From Figure 6.1a, ovotransferrin and ovalbumin were completely digested into oligopeptides (around 37 kDa) and polypeptides (less than 14 kDa) upon hydrolysis with bromelain at all enzyme concentrations. Thus, indicating hydrolysis of EWP occurred and enzyme concentration as little as 0.5% was sufficient to hydrolyse EWP into smaller MW peptides. As the enzyme concentration increased from 0.5 to 4%, the intensity of the protein bands in the 20 kDa and 25 kDa decreased. At 4%, the bands above 25 kDa disappeared, confirming reports in literatures that increasing enzyme concentration resulted in increased degree of protein hydrolysis (Klompong et al., 2007; Chang et al., 2017). Similar result was reported by Chang et al. (2017) who hydrolysed egg white protein with enzyme PC 10F with various enzyme concentrations (0.5, 1, 2 and 4%). They reported reduced intensity in protein bands and increased protein degradation as enzyme concentration increased from 0.5% to 4%.

As shown in Figure 6.1b, hydrolysis of EWP with ficin resulted in the digestion of the high molecular weight (HMW) proteins into polypeptides with a molecular weight (MW) of around 15 kDa and below. With increasing enzyme concentration, intensity of protein bands was reduced and became faint. Subsequently, the digestion pattern of papain was similar to that of bromelain (Figure 6.1a) and ficin (Figure 6.1b) hydrolysis as ovotransferrin and ovalbumin were digested (Figure 6.1c).

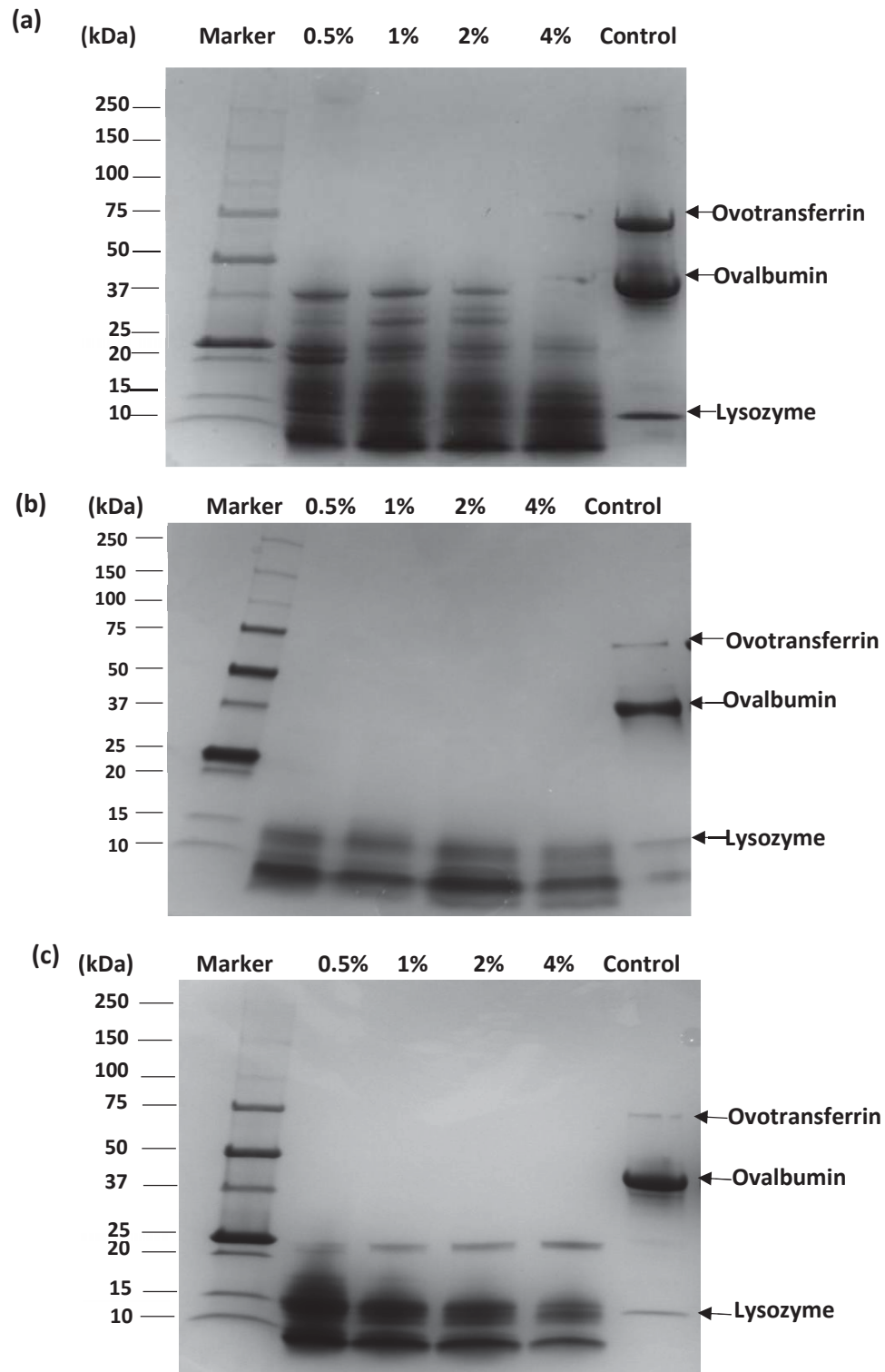


Figure 6.1: SDS-PAGE images of egg white protein hydrolysed with different types of enzymes (a) bromelain, (b) ficin and (c) papain using various amounts of enzymes (0.5, 1, 2 and 4% w/w).

These HMW proteins were digested into peptides below 26 kDa. With increasing enzyme concentration, the intensity of proteins bands around 7 to 25 kDa became light with thin distinguishable bands. The results of EWP hydrolysis indicated that enzyme concentration significantly increased the rate of EWP degradation regardless of the type of enzyme used.

When comparing the rate of EWP degradation among the three enzymes used, it was observed that egg white proteins was digested into MW of around 37 kDa, 15 kDa and 25 kDa by bromelain, ficin and papain, respectively. Therefore, suggesting that ficin degraded EWP more than the other two enzymes, followed by papain. However, the results of DH (Table 6.2) showed papain yielded the highest DH, with no significant difference in the DH of bromelain and ficin after 120 minutes of hydrolysis. Nevertheless, as previously mentioned, comparative studies of enzymes are very complicated because enzymes are specific in their action and have a specific cleavage site (Aspmo et al., 2005; Nimalaratne, Bandara, & Wu, 2005). This may be responsible for the difference between their DH and rate of protein degradation based on molecular weight. Therefore, susceptibility of EWP to enzymatic hydrolysis depends on the type of enzyme used.

To determine the effect of hydrolysis time (0, 30, 60 and 120 minutes) on the proteolytic hydrolysis of EWP, 2% (w/w) enzyme concentration was chosen for each enzyme. As shown in Figure 6.2a, at 1 minute, hydrolysis occurred immediately after mixing EWL containing EWP with bromelain. Ovotransferrin and ovalbumin were digested into smaller peptides of MW around 7 kDa to 37 kDa. As the hydrolysis time increased, the intensity of the protein bands became light and thin. From Figure 6.2b, addition of ficin resulted to immediate digestion of ovotransferrin and ovalbumin into peptides of MW ranging from <10 kDa to 15 kDa at 1 minute of hydrolysis. The intensity of protein bands at higher MW was also observed to decrease, become faint and was digested into small MW with increasing hydrolysis time. Upon addition of papain (at 1 minute), EWP was digested quickly into peptide with MW ranging from <10 kDa to 25 kDa (Figure 6.2b). Intensity of protein bands at higher MW also were reduced with digestion into small MW as hydrolysis time increased. From the results, EWP was immediately digested into lower molecular weight peptides as soon as it was mixed with enzymes. Additionally, increasing hydrolysis time resulted in more egg white protein denaturation. Therefore, enzymatic hydrolysis of EWP was influenced by hydrolysis time.

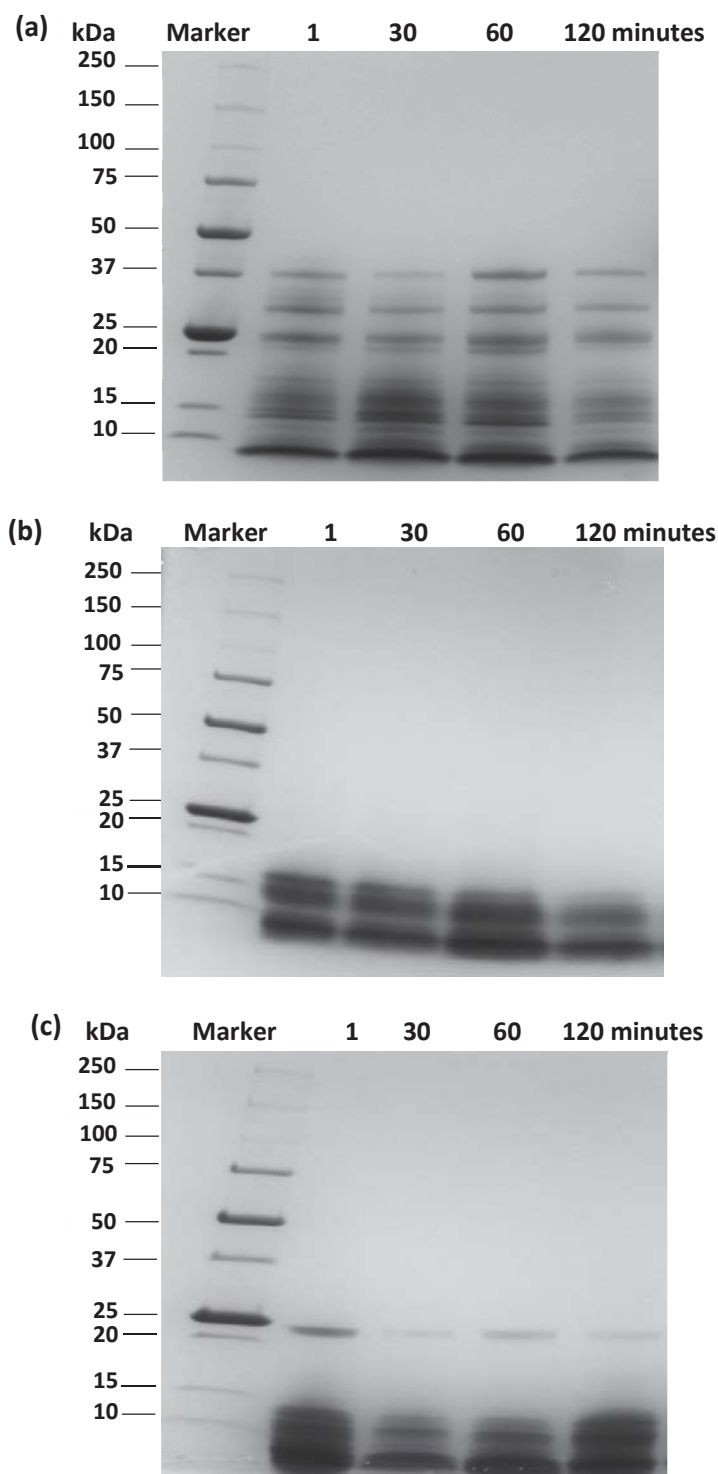


Figure 6.2: SDS-PAGE images of egg white proteins hydrolysed with 2% (w/w) enzyme concentration of (a) bromelain (b) ficin and (c) papain at different time intervals (0, 30, 60 and 120 minutes).

6.4.4 Emulsifying properties of EWPH prepared with bromelain (4% E/S)

6.4.4.1 Characteristics of emulsions

As previously mentioned in Chapter 4, little or no visible droplet aggregates were only produced in emulsions prepared with either 1% (w/w) EWP concentration and oil concentration of $\leq 5\%$ (w/w) or 3% EWP and 1% oil. Additionally, visible droplet aggregates were observed to increase with increasing oil and protein concentrations. In this study, to examine the effectiveness of enzymatic hydrolysis in reducing the formation of large visible droplet aggregates in EWP-stabilised emulsions, some characteristics of emulsions, such as creaming stability, particle size and microstructure of oil droplets in emulsions prepared by original (OEWPH) or hydrolysed egg white protein (EWPH) prepared with 1, 5 and 10% (w/w) EWP and 10% (w/w) oil concentration were investigated. Secondly, a high enzyme concentration of 4% (w/w) was used with the intention of producing EWPH with high DH. The effect of high DH on the emulsifying properties of EWPH was also examined.

It should be noted that the DH of the bromelain egg white protein hydrolysate (BEWPH) using 4% enzyme concentration for preparing emulsion was 5.16%. As seen in Figure 6.3, the EWPH emulsions were brown in colour due to the brown colour of the bromelain enzyme and high E/S (4%) used. Interestingly, as seen in Figure 6.3c, there was a complete absence of visible droplet aggregates in emulsions prepared with EWPH. Even with increase in EWP concentration from 1 to 10% and oil concentration of 10%, no visible droplet aggregates were observed. The reason for the complete absence of aggregates in the emulsions prepared with EWPH could be due to the complete digestion of HMW components of egg white proteins into peptides of LMW as seen from the reducing SDS-PAGE analysis results (Figure 6.2a). Another reason could be due to the high DH (5.16%) of the hydrolysate used to prepare the emulsions. Smaller and smaller peptides are produced with increasing degree of protein hydrolysis which influences the emulsifying properties of proteins (Chobert, Bertrand-Harb, & Nicolas, 1988). During enzymatic hydrolysis, a wide mixture of dipeptides, tripeptide, oligopeptides, polypeptides and free amino acids are produced (Schaafmsa, 2009; El-Salam & El-Shibiny, 2017). The result of this study is interesting as no other studies have reported the use of enzymatic hydrolysis to reduce the formation of visible aggregates in emulsions prepared with EWPH.

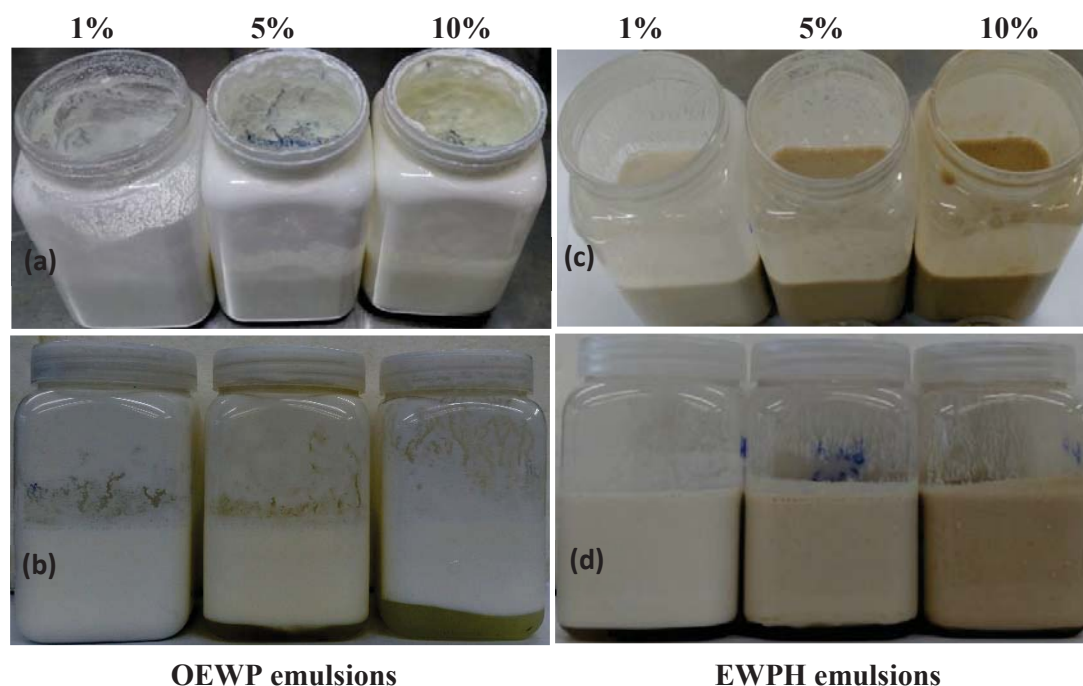


Figure 6.3: Photograph of OEWP and EWPH emulsions prepared with various EWP concentration (1, 5 and 10% w/w) and 10% (w/w) oil. (a) and (c) shows the presence or absence of droplet aggregates (b) and (d) shows presence or absence of phase separation. OEWP and EWPH represents original EWP and EWP hydrolysates, respectively.

However, the photograph in Figure 6.3d showed that, the emulsions prepared with EWPH was less stable to phase separation regardless of the EWPH concentration used. Phase separation was observed to occur immediately after homogenisation, separating into a rich opaque droplet layer floating at the top and a transparent or slightly turbid aqueous layer at the bottom. Phase separation and low emulsifying stability have been reported in whey protein hydrolysates (Turgeon, Gauthier, & Paquin, 1991; van der Ven, Gruppen, d Bont, & Voragen, 2001); potato protein hydrolysates (Cheng, Xiong & Chen, 2010) and monkfish hydrolysates (Greyling, 2017). The mechanism to produce emulsion is attributed to the rapid adsorption of peptides onto the surface of the newly formed oil droplets during homogenisation to form a protective dense layer that inhibits droplets coalescence. Although small peptides rapidly diffuse and adsorb at the interface, they show less efficiency in reducing the interfacial tension since they do not unfold and reorient at the interface like the peptides with HMW (Gbogouri et al., 2004; Klompong et al., 2007).

However, comparing the appearance of the hydrolysed emulsions with those of the control emulsions, emulsions prepared with OEWP exhibited large visible aggregates and the

aggregates was seen to increase with increasing protein concentrations. As previously explained in Chapter 4, the formation of large aggregates could be as a result of depletion flocculation. Depletion flocculation occurs when the concentration of proteins in the continuous phase is above the required protein concentration for full coverage of the newly formed oil droplets. Thus, excess unadsorbed egg white proteins are excluded from the gap between the emulsion droplets at a separation less than that of the protein diameter due to osmotic pressure, causing a net attraction between the droplet and leading to flocculation (Dickson & Golding, 1997; Dickson, 2010). Furthermore, only emulsions containing 1% (w/w) EWP was stable against phase separation, while emulsions containing 5 and 10% (w/w) EWP resulted in aggregation and flocculation which led to phase separation shortly after homogenisation.

The stability of 1% protein indicates that the proteins adsorbed at the oil and water interface was sufficient to cover the newly formed oil droplet (Sanchez & Patino, 2005). Dickson, Golding & Povey (1996) also reported increasing depletion flocculation and phase separation as sodium caseinate concentration increased from 1 to 6% (w/w). Also, they observed that flocculated samples were prone to phase separation rapidly immediately after homogenisation. In summary, enzymatic hydrolysis improved the appearance of emulsions prepared with EWP by preventing the formation of droplets aggregates and flocs even at high protein concentration. Enzymatic hydrolysis was able to break down the HMW egg white proteins into smaller peptides (Klompong et al., 2007).

6.4.4.2 Particle size and size distribution of emulsions

The results of mean particle diameter of emulsions prepared with original and hydrolysed EWP are shown in Table 6.5. The results of particle size ($D_{3,2}$) show that emulsions prepared with EWPH had smaller oil droplet size (0.98-0.66 μm) compared to droplet size of original EWP emulsions (1.22-39.35 μm). Increase in span values with increasing EWP concentration was observed in both types of emulsion. The span values of the control emulsions were higher than those of emulsions prepared with hydrolysed protein. According to Dubey & Parikh (2004), a high span value denotes a high polydispersity and wide size particle distribution. A previous study by Chang et al. (2017) has indicated smaller oil droplet and polydispersity in emulsions prepared with PCF 10F EWP hydrolysate. They attributed it to the LMW of the peptides after hydrolysis. The results also

showed particle size reduced with increasing EWP concentration (1, 5, and 10%) in the emulsion, although no significant difference was observed ($p < 0.05$).

Table 6.5: Particle size and span values of emulsions prepared with various protein concentrations (1, 5 or 10% w/w) and 10% (w/w) oil concentration.

Type of protein	EWP protein (w/w)	D _{3,2} (μm)	Span
OEWP	1	1.22 ± 0.40 ^c	2.15 ± 1.08 ^{ab}
	5	8.73 ± 0.34 ^b	2.44 ± 0.09 ^{ab}
	10	39.35 ± 0.41 ^a	3.05 ± 0.25 ^a
EWPH	1	0.98 ± 0.14 ^c	1.72 ± 0.04 ^b
	5	0.84 ± 0.03 ^c	1.93 ± 0.00 ^{ab}
	10	0.66 ± 0.02 ^c	2.28 ± 0.15 ^{ab}

Each sample results are expressed as mean ± stand deviation of at least two measurements from duplicate experiments. Different letters within a column indicate significant differences at $p < 0.05$. OEWP and EWPH represents original egg white protein and egg white protein hydrolysates respectively.

Protein hydrolysates are surface active and promote the formation of O/W emulsions because they have both hydrophilic and hydrophobic functional groups (Gbogouri et al., 2004; Klompong et al., 2007). Hydrolysates extends their hydrophilic loops into the aqueous phase, while the hydrophobic part extends to the oil phase (Kristinsson & Rasco, 2000).

On the other hand, particle size increased significantly ($p < 0.05$) with increasing EWP concentration in the control emulsions. Increase in protein concentration in the continuous phase causes the viscosity of the continuous phase to increase, which can slow down the migration of the proteins unto the interface to be adsorbed by the oil droplets (Dagorn-Scaviner, Gueguen, & Lefebvre, 1987; Guo & Mu, 2011). Protein concentration is an important parameter because it influences an emulsion droplet size, storage stability and surface protein concentration (Srinivasan, Singh & Munro, 1996; Hu, McClements & Decker, 2003). From the results obtained, smaller droplet size was obtained in emulsions prepared with EWPH than those prepared with OEWP. Additionally, there was no significant difference in droplet size with increasing EWPH concentration. However, droplet size increased significantly with increasing OEWP concentration. The span values

of the OEWP emulsions were higher than those obtained for the EWPH emulsions. In summary, enzymatic hydrolysis improved the particle size of emulsion droplets and emulsifying properties of EWPH. Enzymatic hydrolysis of protein affects the molecular size, results in exposure of buried hydrophobic peptides as well as ionizable and polar groups which are important for interfacial and emulsifying properties (Mutilangi et al., 1996; Rahali et al., 2000).

As shown in Figure 6.4a, particle size distribution (PSD) of the emulsions prepared with EWPH were similar, narrow and bimodal at all EWPH concentration (1, 5 and 10% w/w) used. On the other hand, for the control emulsions, the PSD was broader and only emulsion containing 10% EWP was monomodal while the PSD for the other two emulsions (1 and 5%) emulsions were bimodal (Figure 6.4b).

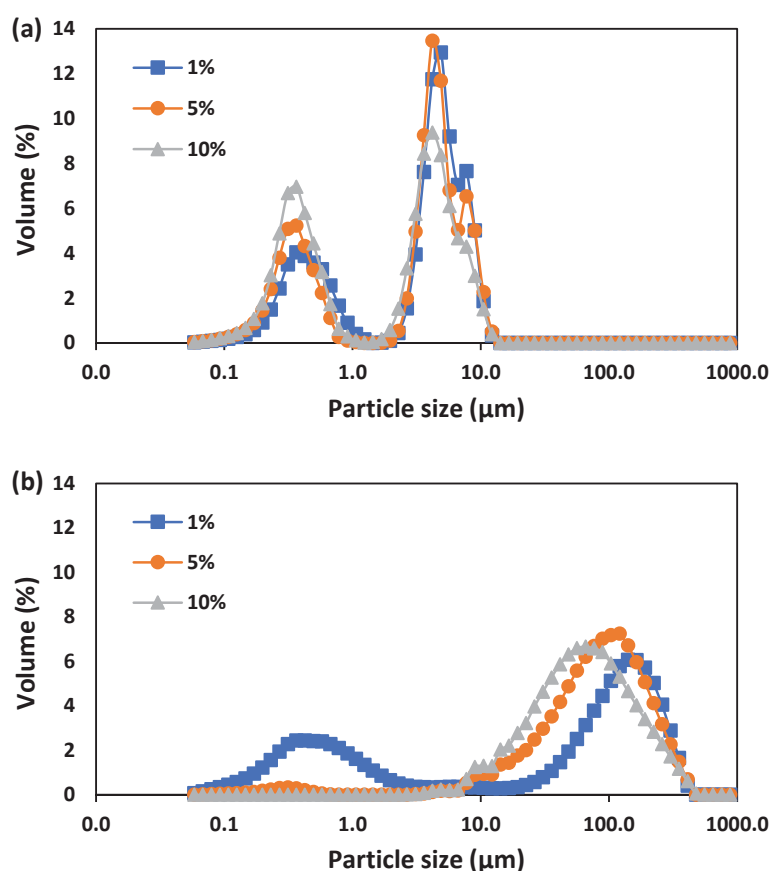


Figure 6.4: Particle size distributions of emulsions prepared with various EWP concentrations (1, 5 and 10% w/w) and 10% oil concentration. (a) original egg white protein emulsion (b) egg white protein hydrolysate emulsion.

At 5%, a very smaller second peak was observed compared to the second peak of 1% EWP emulsion. The differences in the PSD of the control emulsions shows that EWP concentration influenced their particle size as seen from the droplet size result (Table 6.5). The similar PSD observed for the hydrolysed emulsions indicates that EWPH concentration did not influence the particle size of the hydrolysed emulsions. This was in accordance with the results of the particle size analysis (Table 6.5).

6.4.4.3 ζ - potential of emulsions

The ζ -potential results of emulsions prepared with OEWP and EWPH are shown in Figure 6.5. ζ -potential ranged from -15.0 to -28.7 mV for the hydrolysed emulsions and -32.3 to -35.9 mV for the control emulsions. ζ -potential of both hydrolysed and control emulsions was seen to decrease with increasing protein concentration. When comparing the ζ -potential of emulsions prepared from OEWP and EWPH. OEWP emulsions possessed a higher ζ -potential than those obtained for EWPH ($p < 0.05$). Generally, ζ -potential values of ≤ -30 mV or $\geq +30$ mV are required to prevent droplet aggregation and increase emulsion stability in emulsions (Achouri, Zamani & Boye, 2012; McClements, 2015). With reference to this theory, this could explain the susceptibility of EWPH emulsions to phase separation immediately after homogenization. Suggesting peptides produced by hydrolysis were not efficient in reducing the interfacial tension since they were not flexible enough to orient at the interface like large peptides (Gbogouri et al., 2004). Thus, there was an absence of strong electrostatic repulsion between the oil droplets (Chanamai & McClements, 2002).

Nevertheless, on this basis of this theory, emulsions prepared with original EWP should be stable against aggregation, creaming or flocculation for a long period of time. However, only emulsions prepared with 1% OEWP was stable against separation. Thus, indicating enough proteins were adsorbed on the surface oil droplets, conferring electrostatic charge needed to maintain emulsion stability. However, despite the high ζ -potential values (-32.3 mV and -35.7 mV) of emulsions stabilised by 5 and 10% OEWP, phase separation still occurred. According to Thamnarathip et al. (2016), stability of emulsions against aggregation, creaming and flocculation does not always correlate with high zeta potential if the oil droplet particle size is too big to prevent creaming, suggesting other factors aside ζ -potential was involved in emulsification and stability.

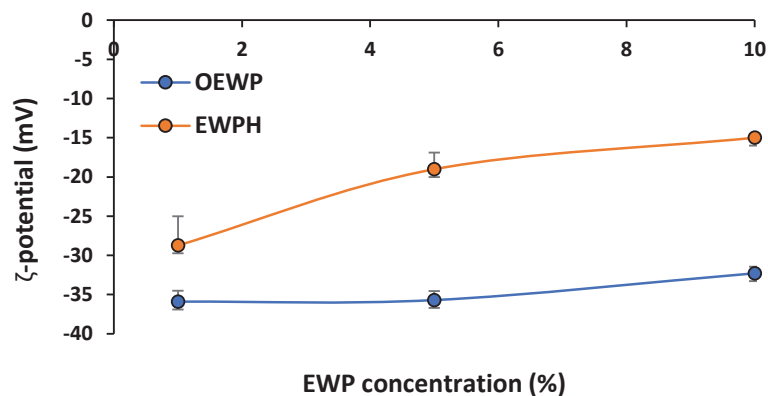


Figure 6.5: Mean ζ potential of emulsions prepared with various protein concentrations and 10% (w/w) oil concentration. OEWP and EWPH represents original egg white protein and egg white protein hydrolysates respectively. Data points represent the means $\pm$ SD (n =6).

6.4.4.4 Microscopic examination of emulsions

To better illustrate the nature of oil droplets in emulsions prepared with original and hydrolysed egg white protein, the microstructure of the emulsions was investigated as shown in Figure 6.6. For the emulsions prepared with EWPH, the microstructure shows droplet aggregation which progressed extensively as EWPH concentration increased from 1 to 10%. Nevertheless, the results do not agree with the particle size results and visual appearance. From the visual appearance of the emulsions, no sign of droplet aggregation or flocculation was observed compared to those of the control emulsions with lots of aggregates/flocs. Additionally, particle size of 0.66 to 0.98 μm was reported. However, the extensive droplet aggregation and flocculation observed could be as a result of their low ζ -potential values which caused a decrease in the electrostatic repulsion between the emulsion droplets leading to aggregation and flocculation (McClements, 2004).

In comparison, the microstructure of emulsions prepared with original EWP showed smaller droplet size and more stability against droplet aggregation and flocculation at 1% EWP. This may be attributed to sufficient proteins adsorbed at the interface to form protective layer around the individual droplets and was responsible for its stability against phase separation (McClements, 2004). Secondly, the emulsion must have benefitted from electrostatic repulsion force associated with their high ζ -potential (-35.9 mV) (McClements, 2015). When the protein concentration increased 5% and 10%, extensive droplet aggregation and flocculation was observed and was highest at 10%. This may be

attributed to depletion flocculation caused by excess unadsorbed proteins in the continuous phase, causing the proteins to be depleted from the gaps between the oil droplets by osmotic pressure. Thereby causing a net attraction among the oil droplets and formation of droplets floc (Dickson, 2010). The microstructure results obtained was in agreement with the mean particle size and visual appearance of the emulsions, which showed increasing droplets aggregation with increase in EWP concentration.

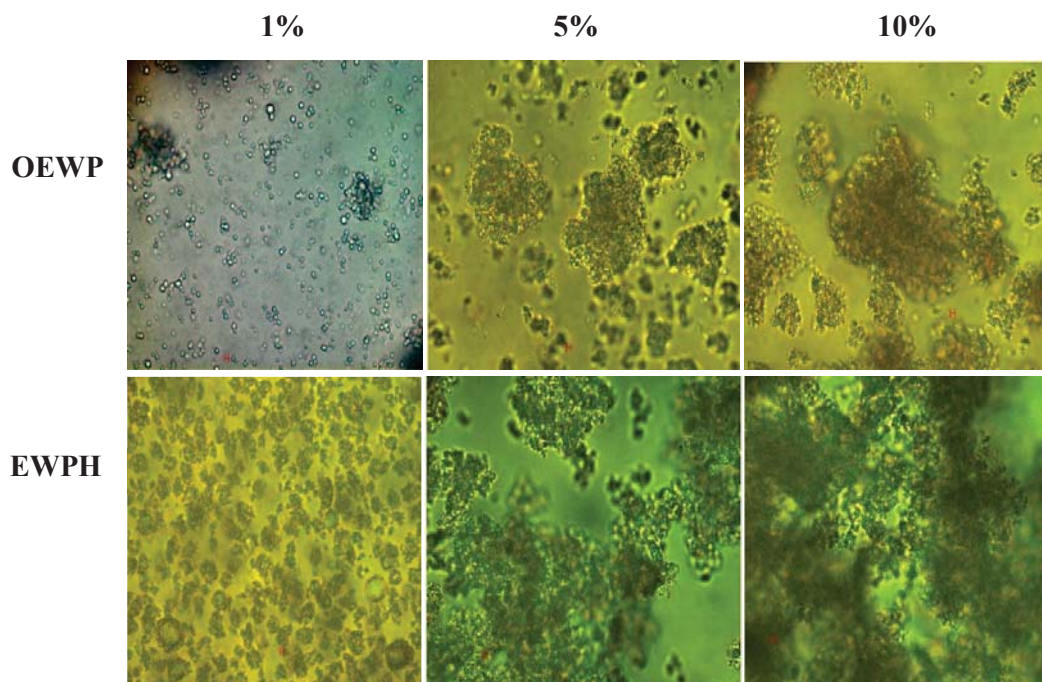


Figure 6.6: Microscopic images of control and hydrolysed emulsions prepared with various concentration of EWP (1, 5 and 10% w/w) and 10% (w/w) oil. OEWP and EWPH represents original egg white protein and egg white protein hydrolysates respectively. The scale bar inserted represents 20 μm .

6.4.4.5 Colour of emulsions

Due to the colour difference between the emulsions prepared with original EWP and EWP hydrolysates, the colour pattern between emulsions at different EWP concentration was investigated and is shown in Figure 6.7. From Figure 6.7a, at the same EWP concentration, the OEWP emulsions showed a higher *L* value (whiter emulsion) than the EWPH emulsions. This could be attributed to the brown colour of bromelain enzyme used for hydrolysis. The type and colour of emulsifier used in emulsion making play a major impact on the lightness and colour of emulsions produced (Chung, Sher, Rousset, Decker, & McClements, 2017).

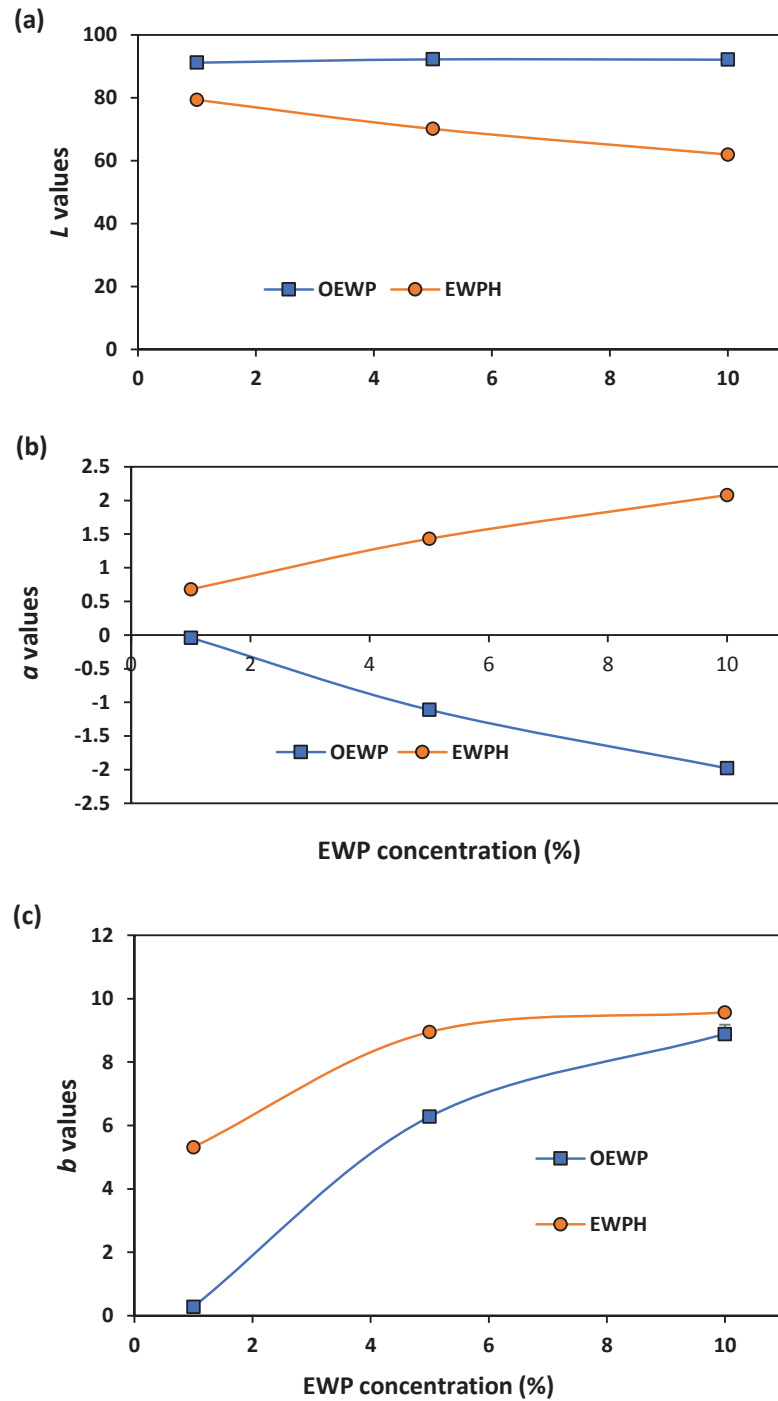


Figure 6.7: Colour specifications (L, a, b values) of emulsions prepared with original and hydrolysed egg white proteins. OEW and EWP represents original egg white protein and egg white protein hydrolysates respectively. Data points represent the means $\pm$ SD (n =6).

For the control emulsion, no significant difference ($p < 0.05$) in lightness was observed with increased OEW concentration. However, significant difference in lightness ($p < 0.05$)

was found among the hydrolysed emulsions with lightness reducing (emulsions became darker) with increased EWP concentration. This was expected as the emulsion prepared with 10% (w/w) EWP had a very dark colour compared to the others because the EWP containing 10% (w/w) was diluted to obtain 1 and 5% (w/w) EWP concentrations (See Figure 6.3). This decrease in lightness in the hydrolysed emulsions could be attributed on the selective absorption by the emulsifier meaning that their surfaces reflected less light because of reduced back-scattered light intensity and lightness (McClements, 2002).

As shown in Figure 6.7b, the emulsions prepared with EWP had positive *a*- values (red coordinate) indicating they had a reddish tinge, whereas the emulsions prepared with OEWP showed negative *a*- values (green coordinates) indicating a greenish tinge (Figure 6.7b). Both type of emulsions showed positive *b*- values indicating yellowish tinge across all concentration of EWP used (Figure 6.7c). The *a*- and *b*- values for both type of emulsion increased with increasing EWP concentration. This differences in colour should be taken into consideration when formulating emulsions from bromelain hydrolysed EWP (due to the brown colour) as consumers have an expected appearance of emulsions.

6.4.4.6 Effect of heat treatment on EWP emulsions

Generally, food emulsions are subjected to heat treatment to extend their shelf life (Keowmaneechai & McClements, 2006). However, studies have shown post-homogenisation heat treatment have a profound effect on the droplet size, microstructure and rheological properties of protein-stabilized emulsions (McSweeney et al., 2004; Liang et al., 2013). To determine the thermal stability of emulsions prepared with EWP, emulsions containing 10% protein was chosen and diluted to 1 and 5% protein concentration. The emulsions containing 1, 5 and 10% protein concentration were then subjected to various temperatures 60, 65, 70, 80 and 90°C at 0 and 30 minutes. As shown in Figure 6.8, heat-treatment resulted in loss of stability in the EWP emulsions containing EWP concentration (1, 5 and 10%) at all temperature and time used. At 5% gelation occurred at all temperatures except at 60 and 65°C at 0 minute where visible droplet aggregation could be seen. This instability at 5 and 10% EWP could be due to their high protein concentration. Liang et al. (2013) reported that emulsions containing high protein concentration produce gels upon heat treatment. At 1% EWP, visible droplet aggregation and phase separation was observed at all temperature and time. indicating hydrolysed emulsions were highly susceptible to heat treatments.

Singh & Dalgleish (1998) reported some sort of destabilization of whey protein hydrolysate emulsions thermally treated at 90°C for 30 minutes and at 120°C for 15 minutes. Similar destabilisation result has been reported in whey protein oil-in-water emulsions containing hydrolysed lecithin after heat treatment (Liang et al., 2013). The authors attributed this instability to the reduced efficiency of the LMW peptides produced during hydrolysis to form thick protective layer around the newly formed droplets and strong electrostatic repulsion force among the droplets, causing destabilisation upon heat treatment.

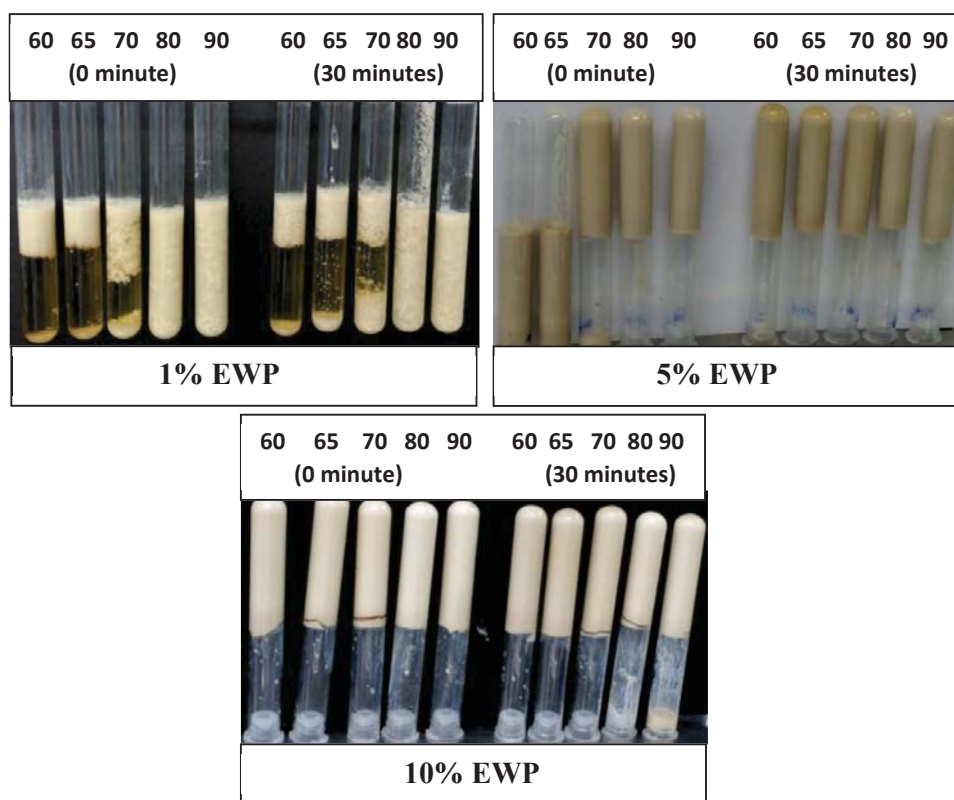


Figure 6.8: Photograph of thermal treatment of EWP emulsions prepared with various EWP concentration (1, 5 and 10% w/w) and 10% (w/w) oil.

Globular proteins such as EWP are easily denatured by heat and it is well known that EWP denatures at around 60 to 62°C (Belitz et al., 2009; Lechevalier et al., 2017). Denaturation causes the protein molecules to unfold and form aggregates and if the concentration is quite high, gels can be formed which can be desirable in forming textures (e.g. formation of whey protein emulsion gel matrix) (Dickson, 2012) or undesirable (Dalgleish, 1997; Akkouche,

Aissat, & Madani, 2012). Studies have shown small protein peptides are readily exposed upon heating, however they are not efficient in prevent droplet aggregation and coagulation because they lack strong electrostatic and steric protective layer (Euston, Finnigan, & Hirst, 2000; Ye, Hemar, & Singh, 2004; Ye & Singh, 2006). The results obtained indicates poor stability of emulsions prepared with hydrolysed egg white proteins due to lack of strong steric protective layer provided by the peptides for the emulsion droplets.

To further illustrate the effect of thermal treatment on droplet size, particle size was measured in emulsions containing 1% EWP. Particle size could not be measured on emulsions containing 5 and 10% EWP because of gelation of the emulsions. As shown in Figure 6.9, particle size increased with increasing temperature and time. Visually (Figure 6.8) there was no difference in droplet instability with the treatment time. In general, droplet size of protein stabilised emulsions increases after heat treatment and the extent of aggregation and coagulation is time and temperature dependent (McSweeney et al., 2004). Increase in size as a result of heat may be due to protein denaturation and formation of micro-gel, as thermal treatment causes more exposure of hydrophobic groups to the surface of protein structure which influences their interfacial adsorption properties (Chang et al., 2016). Secondly, high temperatures cause more proteins to spread on the emulsion droplets leading to increase in size (Dybowska, 2008).

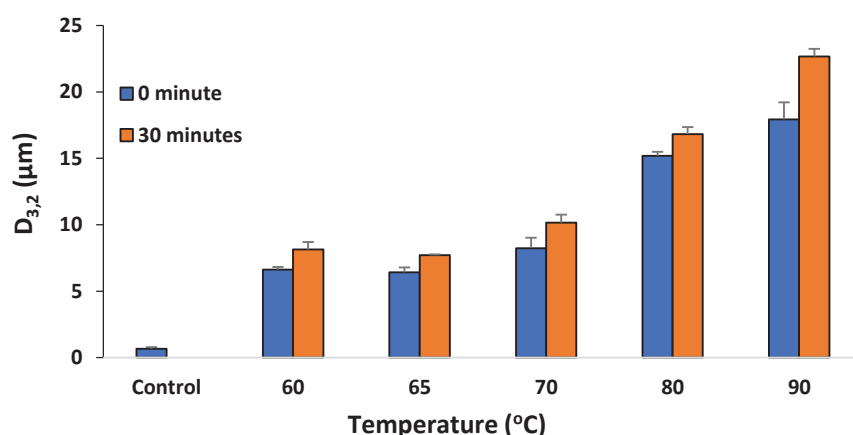


Figure 6.9: Particle size diameter (D_{3,2}) of EWPH emulsions prepared with 1% (w/w) EWP and 10% (w/w) oil heat treated at various temperature (60, 65, 70, 80 and 90°C) for 0 and 30 minutes. Data points represent means $\pm$ SD (n =6).

6.4.5 Emulsifying properties of EWPH prepared with 1% (w/w) bromelain and ficin

6.4.5.1 DH

The degree of hydrolysis of EWPH samples prepared with 1% (w/w) (accounted for weight ratio of EWP) of ficin and bromelain after 2 and 4 hours hydrolysis is shown in Figure 6.10a. The DH measures the degree of a protein's hydrolytic breakdown, higher DH denotes increased breakdown of proteins (Cho et al., 2014; Noh & Suh, 2015). From Figure 6.10, it was observed that increasing hydrolysis time from 2 to 4 hours, increased DH significantly from 4.10 to 4.87% for ficin hydrolysate and from 4.03 to 4.96% for bromelain hydrolysate. Similar increase in DH (34.97 to 61.96%) was also reported in sturgeon viscera hydrolysates prepared with alcalase when hydrolysis time increased from 30 to 204 minutes (Ovissipour et al., 2009). Klompong et al. (2007) also observed increase in DH in yellow stripe trevally hydrolysate prepared with different concentrations (0.25, 0.5, 1, 2.5, 5, 7.5 and 10% w/w) of alcalase and flavouzyme as hydrolysis time increased from 0 to 20 minutes. From the result bromelain and ficin treatments showed similar DH levels after 2 and 4 hours hydrolysis, indicating enzyme type did not significantly affect DH of EWPH. The results obtained indicates higher DH levels can be obtained by increasing hydrolysis time, also DH was not affected by enzyme type.

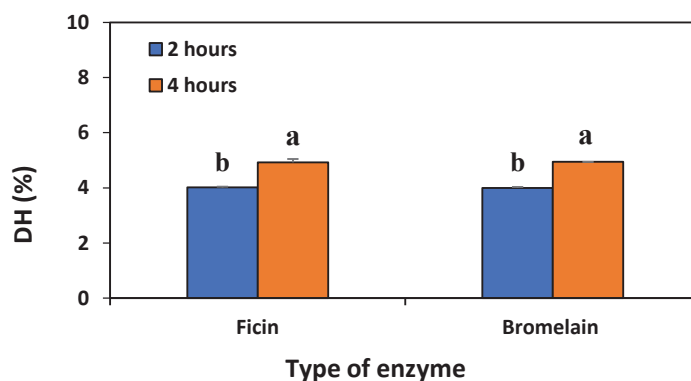


Figure 6.10: DH of egg white protein hydrolysates prepared with bromelain and ficin after 2 and 4 hours. Results are expressed as means $\pm$ SD of two independent replicates. ^{a,b} Means with different superscripts are significantly different ($p < 0.05$).

6.4.5.2 Particle size and ζ -potential

The effect of enzyme type (ficin and bromelain) and hydrolysis time (2 and 4 hours) on the emulsifying properties of EWPH emulsions containing 10% (w/w) oil and 1% (w/w) EWP

was studied. Also, a lower enzyme concentration of 1% was chosen for both hydrolysis to examine its effect on the emulsifying property of EWPH. As shown in Figure 6.11a, the particle size of ficin EWPH emulsions (FEWPH) ranged from 0.75-0.87 μm and 6.4 to 7.37 μm for emulsions prepared with bromelain EWPH (BEWPH). Particle size was observed to decrease with increasing hydrolysis time for both FEWPH and BEWPH. Although, hydrolysis time did not significantly ($p < 0.05$) affect the particle size in FEWPH emulsion. Significant difference ($p < 0.05$) was observed in the particle size in BEWPH emulsion with increase in hydrolysis time.

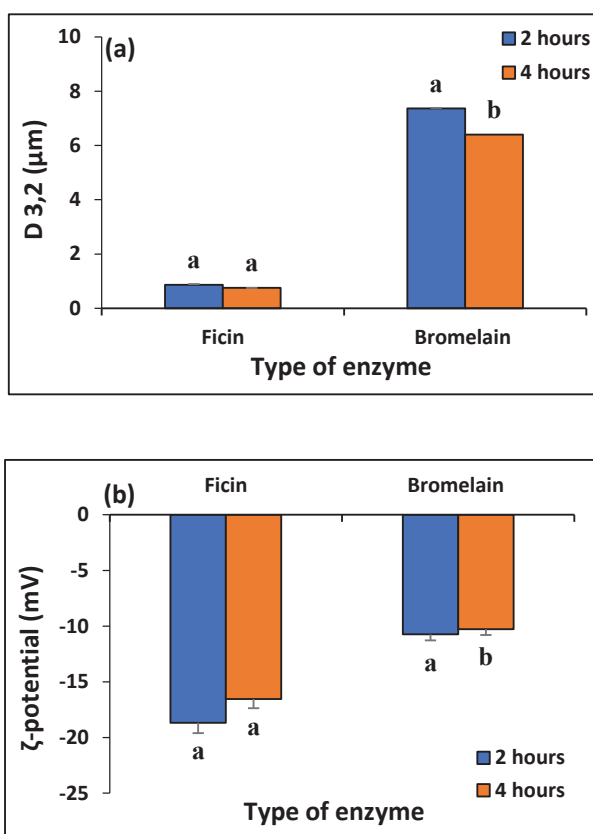


Figure 6.11: (a) Particle size diameter (D_{3,2}) and (b) ζ -potential of emulsions prepared with ficin and bromelain EWPH (at an E/S ratio of 1% w/w) hydrolysed for 2 and 4 hours containing 1% (w/w) EWP and 10% oil. FEWPH and BEWPH represents ficin egg white protein and bromelain egg white protein hydrolysates respectively.

Data points represent means $\pm$ SD of duplicate measurements for two replications. ^{a,b} Means with different superscripts are significantly different ($p < 0.05$).

When comparing the particle size of FEWPH emulsion with that of BEWPH emulsion, it was observed that FEWPH produced smaller droplet size than those of BEWPH. Klompong

et al. (2007) also reported significant difference in emulsifying properties of alcalase and flavourzyme hydrolysate. According to Klompong et al. (2007), the emulsifying properties of emulsion produced with different enzymes may be different due to different sequence and amino acid composition in their peptides. Each enzyme is different in its action because of its unique specificity (Kristinsson & Rasco, 2000; El-Salam & S. El-Shibiny, 2017). As shown in Figure 6.11b, ζ -potential values ranged from -16.55 to -18.68 mV for the FEWPH emulsions and -10.75 to -10.29 mV for the BEWPH as hydrolysis increased from 2 to 4 hours. The FEWPH emulsions showed higher ζ -potential values than those of BEWPH emulsions (Figure 6.11b). The results agreed with the particle size results, confirming the smaller particle size in FEWPH emulsions compared to those of BEWPH emulsions.

When compared to the droplet size of the emulsions prepared using 4% BEWPH prepared with 10% oil and 1% EWP (Table 6.5) with those of 1% BEWPH at the same oil and protein concentration at 2 hours hydrolysis time. The results revealed smaller particle size when the bromelain enzyme concentration was 4% (0.98 μm) compared to the 6.4 μm obtained using 1% bromelain concentration. This could be attributed to the higher DH value (5.16%) obtained using 4% bromelain compared to the 4.03% DH after 2 hours hydrolysis. Similar result was reported by Euston et al. (2001), who observed increased emulsifying ability of whey protein hydrolysates with increase in DH from 10-27%. Contrary result was reported by Chen et al. (2012), authors reported reduced emulsifying capacity and ability as DH increased from 5.2-14.7% in trypsin EWP. Although, emulsions produced using 1% bromelain had larger droplet size, they did not separate immediately after homogenisation unlike emulsions prepared with 4% bromelain emulsions which separated immediately they were produced. An extensive hydrolysis or extremely high DH may result in the production of free amino acids and lower molecular weight peptides (Kristinsson & Rasco, 2000; Euston et al., 2001). Peptides with very LMW do not have the flexibility to orient at the interface like the peptides with HMW, which can reduce its emulsifying ability (Kristinsson & Rasco, 2000; Klompong et al., 2007). As a consequence, the emulsion stability of 4% bromelain was susceptible to phase separation. Thus, a careful selection of hydrolysate with reasonable DH level is important for EWP emulsion stability.

As shown in Figure 6.12, both FEWPH and BEWPH emulsions exhibited a bimodal size distribution at 2 and 4 hours hydrolysis time. Nevertheless, a relatively higher proportion of large droplets was observed in the BEWPH emulsions, while a proportion of smaller droplets around 1 μm could be observed in the FEWPH emulsions. The results obtained

collaborated well with the particle size results (Figure 6.11a). In both type of emulsions, the PSD of 2 hours emulsion appeared similar to those of 4 hours emulsion.

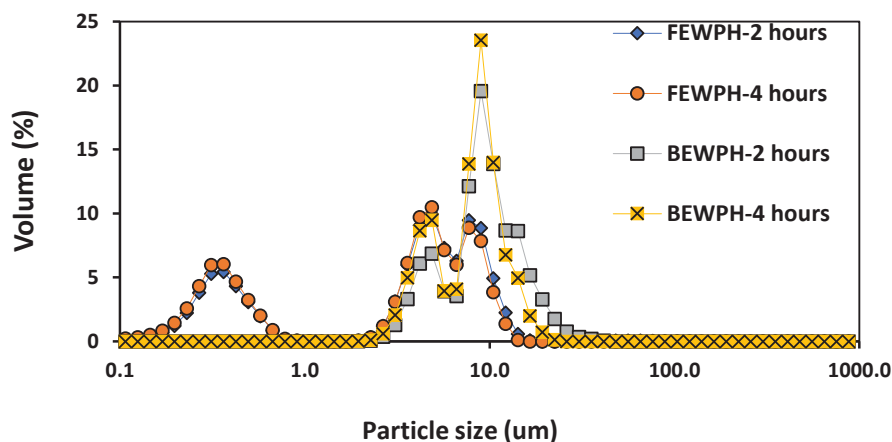


Figure 6.12: Particle size distribution of emulsions prepared with prepared with ficin and bromelain EWP (at an E/S ratio of 1% w/w) hydrolysed for 2 and 4 hours containing 1% (w/w) EWP and 10% oil. FEWPH and BEWPH represents ficin egg white protein and bromelain egg white protein hydrolysates respectively.

Data points represent means $\pm$ SD of at least duplicate measurements from two independent experiments.

6.4.5.3 Visual appearance and creaming stability

As shown in Figure 6.13, the emulsions prepared with 1% ficin and bromelain EWP hydrolysed for 2 and 4 hours contained no visible aggregates and were similar to those obtained with 4% BEWPH. From Figure 6.14, it was seen that the emulsions showed no signs of phase separation on the day of preparation. However, the following day, emulsions separated into rich opaque droplet layer at the top and a transparent aqueous serum layer at the bottom. More phase separation was observed in the BEWPH emulsions than in the FEWPH emulsions. This could be attributed to the larger droplet size and lower ζ -potential value observed in the BEWPH emulsions. Visually, little difference was seen in the appearance of FEWPH and BEWPH emulsions prepared after 2 or 4 hours.

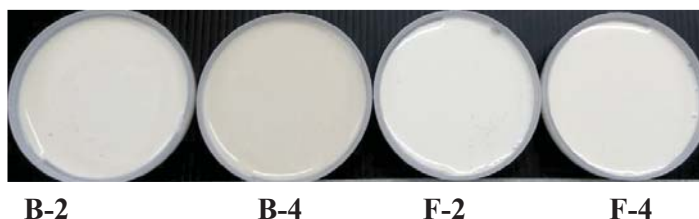


Figure 6.13: Photograph showing appearance of FEWPH and BEWPH emulsions. B-2 = BEWPH after 2 hours, B-4= BEWPH after 4 hours, F-2= FEWPH after 2 hours, F-4= BEWPH after 4 hours.

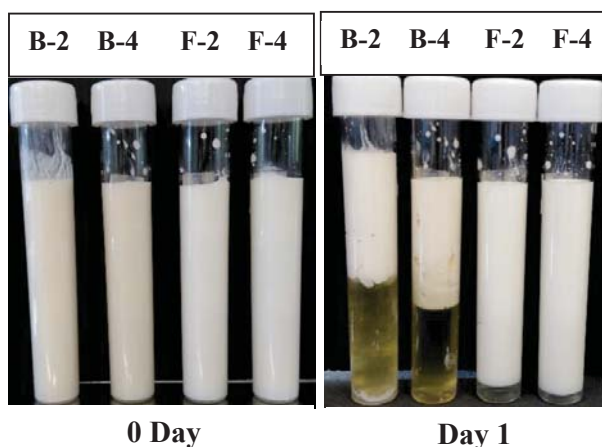


Figure 6.14: Photograph showing the onset of phase separation appearance in FEWPH and BEWPH emulsions hydrolysed for 2 of 4 hours. B-2 = BEWPH after 2 hours, B-4= BEWPH after 4 hours, F-2= FEWPH after 2 hours, F-4= BEWPH after 4 hours.

6.4.5.4 Microscopic examination of emulsion

As shown in Figure 6.15, droplet aggregation was observed in both emulsions prepared with FEWPH and BEWPH. However, lesser aggregation and smaller droplet size was observed in the FEWPH emulsions than the BEWPH emulsions. The microstructure results agreed with the particle size result which reported smaller particle size for FEWPH emulsions and larger droplet size for BEWPH emulsions. The large particle size (See Table 6.5) observed from the particle size results could be attributed to the aggregated emulsion oil droplets observed in the microstructure. As already stated, the low ζ -potential value obtained contributed to its large droplet size due to lack of strong electrostatic repulsion between the emulsion droplets, causing them to form aggregates (McClements, 2004). The microstructure image also confirms that hydrolysis time did not significantly affect the emulsion droplet size, since microstructure images obtained for the emulsions prepared with ficin and bromelain EWPH hydrolysed after 2 and 4 hours looked similar. From the results of the microscopic examination, ficin EWPH produced smaller droplet size than

those prepared with bromelain EWPH. Thus, enzyme type influenced the droplet size of EWPH emulsions than hydrolysis time.

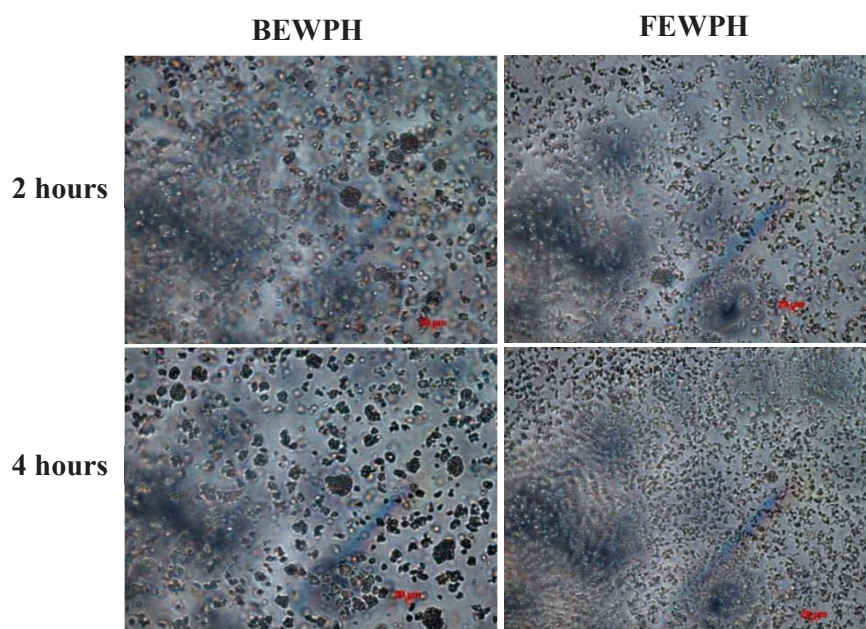


Figure 6.15: Microstructure of emulsions prepared with ficin and bromelain EWPH (at an E/S ratio of 1% w/w) hydrolysed for 2 and 4 hours containing 1% (w/w) EWP and 10% oil. FEWPH and BEWPH represents ficin egg white protein and bromelain egg white protein hydrolysates respectively.

6.4.5.5 Colour measurement

As shown in Figure 6.13, the emulsions prepared with 1% BEWPH had a slight brown colour as opposed to the deep brown colour of the 4% BEWPH emulsions (See Figure 6.3). The emulsions prepared with 1% FEWPH was white in colour. Reducing the enzyme concentration from 4% to 1% significantly reduced the deep brown colour obtained at high E/S ratio. Colour measurement was carried out to evaluate the colour difference between the ficin and bromelain hydrolysate emulsions. As shown in Table 6.6, lightness decreased with increase in hydrolysis time in both type of emulsions. When compared, the BEWPH emulsions had a higher L than FEWH emulsions. a -values was positive (red colour) in both emulsions with BEWPH emulsions having higher a -value (more red colour) than FEWPH, which was expected due to bromelain brown colour. Additionally, positive b -values (blue

colour) was observed in both type of emulsion with *b*-value increasing with increase in hydrolysis time. Comparing the colour values of 1% BEWPH (hydrolysed for 2 hours, See Table 6.5) and that of 4% BEWPH (hydrolysed for 2 hours, See Figure 6.7) The *L* value of 1% Bromelain EWH (94.8) was much higher (lighter) than those obtained for 4% Bromelain EWH (61.9), while *a*-value (0.26) and *b*-values (8.27) were lower than those of 4% Bromelain EWH (2.08 and 9.57) meaning they were less red and or blue. This could also be seen from their visual appearance (See Figure 6.3 and 6.12). The results of obtained shows enzyme type and hydrolysis time significantly ($p < 0.05$) affected emulsion colour.

Table 6.6: Specifications of *L*, *a*, *b* colour values of FEWPH and BEWPH emulsions hydrolysed for 2 and 4 hours.

Type of emulsion	<i>L</i>	<i>a</i>	<i>b</i>
FEWPH-2 hours	94.8 ± 0.37 ^b	0.26 ± 0.01 ^c	8.27 ± 0.06 ^c
FEWPH-4 hours	90.1 ± 0.09 ^c	0.13 ± 0.01 ^d	9.49 ± 0.01 ^a
BEWPH-2 hours	96.4 ± 0.03 ^a	0.39 ± 0.01 ^b	8.04 ± 0.01 ^d
BEWPH-4 hours	95.6 ± 0.08 ^b	0.49 ± 0.01 ^a	8.83 ± 0.01 ^b

Means ± SD of triplicates of duplicate measurements from two independent samples. FEWPH and BEWPH represents ficin egg white protein and bromelain egg white protein hydrolysates respectively. Means with different letters within a column are significantly different ($p < 0.05$).

6.4.6 Characteristics of 0.3% FEWPH emulsions

Following the higher emulsifying ability and smaller droplet size obtained using 1% E/S ratio of ficin (Figure 6.11), the enzyme concentration of ficin was further reduced to 0.3% and hydrolysis was carried out for 4 hours. As seen in Figure 6.16, the 0.3% FEWPH emulsion showed no separation on the day of preparation, but showed slight separation (rich opaque droplet layer at the top and a transparent aqueous serum layer at the bottom) the following day. The results of Table 6.7, clearly shows the 0.3% FEWPH had a DH of 3.01%, emulsion particle size of 1.27 µm and ζ-potential value of -8.48 mV.

Furthermore, the particle size obtained for 0.3% FEWPH (1.27 µm) were higher than those prepared with 1% FEWPH (0.87 and 0.75 µm hydrolysed for 2 and 4 hours respectively). Additionally, its ζ-potential value was low (-8.48 mV) compared to those of 1% FEWPH (-16.93 and -19.21 mV). The reason for the large particle size of the 0.3% FEWPH could be attributed to insufficient peptides available to completely cover the surface of the oil droplets, causing the droplets to re-coalesced and flocculate, resulting in larger droplet size

(Qian & McClements, 2011). The DH obtained was low at 0.3% E/S ratio (3.01%), a low DH indicates less amount of proteins broken down into peptides, meaning insufficient protein peptide available (Gauthier et al., 1993). The results indicate that an increased E/S ratio and DH can improved droplet size as more peptide will be available and cause the hydrophobic amino acids of the hydrolysates to be more exposed (Horax, Vallecios, Hettiarachchy, Osorio, & Chen, 2017).

However, an extensive hydrolysis or extremely high DH may cause an unbalance between the hydrophobic and hydrophilic groups of a protein hydrolysate (Horax et al., 2017). Several studies have reported undesirable emulsifying property in whey protein hydrolysates with DH > 20%. (Agboola, Singh, Munro, Dalgleish, & Singh, 1998; Dalgleish & Singh, 1998; Scherze & Muschiolik, 2001). Nevertheless, a very mild hydrolysis or low DH can cause protein aggregation, which if too big may lead to bridging flocculation (Euston et al., 2000). An important criterion for improved emulsifying property of hydrolysates depends on a controlled DH (van der Ven et al., 2001; Euston et al., 2001; Horax et al., 2017).

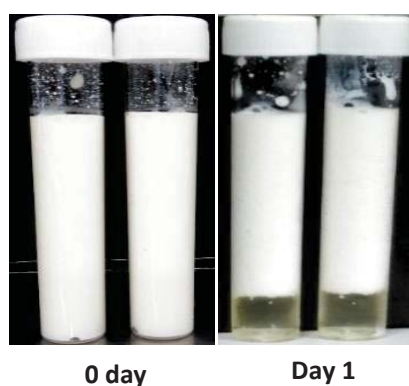


Figure 6.16: Photograph of 0.3% EWPH emulsions showing no separation on the day of preparation and phase separation the next day.

Table 6.7: Emulsion properties of emulsions prepared from 0.3% FEWPH containing 10% (w/w) oil and 1% EWP. Data are presented as mean and standard deviation (n =4)

Emulsion properties	D _{3,2} (μm)	ζ-potential (mV)	DH (%)
0.3% FEWPH	1.27 ± 0.03	-8.48 ± 0.34	3.01 ± 0.03

6.5 Conclusions

This present study investigated the effects of enzyme type, enzyme concentration and hydrolysis time on DH and emulsifying properties of EWP hydrolysates prepared with three different proteolytic enzymes (bromelain, ficin and papain). DH was positively influenced by enzyme concentration and hydrolysis time for the three enzymes. However, significant difference among the enzymes was observed at 4% E/S ratio at 120 minutes with papain yielding the highest DH, while similar DH level was observed for ficin and bromelain. SDS-PAGE analysis showed enzyme concentration and hydrolysis time affected the digestion process and EWP's major components, ovotransferrin and ovalbumin, were completely hydrolysed into smaller peptides at the end of the hydrolysis process. Importantly, this study has demonstrated that enzymatic hydrolysis can completely remove droplet aggregates and flocs usually formed in emulsions prepared with original EWP. The emulsion prepared with EWPH (4% E/S) generated smaller emulsion droplet size than those prepared with OEWP regardless of the EWP concentration used. However, the emulsions were prone to phase separation immediately after homogenisation probably due to extensive hydrolysis (high DH) reducing the efficiency of the LMW peptides to form dense protective layer around the emulsion droplets inhibiting aggregation and separation. The emulsions prepared with hydrolysed proteins were susceptible to thermal degradation at all temperatures (60, 65, 70, 80 and 90°C) and time (0 and 30 minutes) used regardless of the protein concentration. Phase separation was also observed with bromelain (1% E/S) and ficin (0.3% and 1% E/S) hydrolysed for 2 and 4 hours. However, phase separation occurred the following day after preparation. Droplet size and ζ -potential of emulsions were affected by enzyme type and DH. 4% BEWPH (DH 5.16%) yielded smaller droplet size than those of 1% BEWPH (DH 4.10% and 4.87%). The same phenomenon was observed for FEWPH, as 1% FEWPH (DH 4.03% and 4.96%) yielded smaller droplet size than that of 0.3% FEWPH (DH 3.01%). This study provided useful information for the design and use of hydrolysates from EWP as emulsifiers in food emulsions.

Chapter 7. Overall Conclusions & Recommendations

The comprehensive results obtained in this research focussed on developing stable egg white protein (EWP) emulsions prepared with egg white liquid (EWL) with little or no aggregates. This thesis comprised of three main parts, the first part focused on the effects of pH and heat treatment on protein aggregation and partial protein denaturation of egg EWP; the second part investigated the effects of various factors, such as heat treatment, oil concentration and protein concentration, on the reduction of large visible aggregates formed in emulsions prepared with EWL containing different concentrations of EWP and the third part studied the effect of enzymatic hydrolysis on the degree of hydrolysis and emulsifying properties of EWP hydrolysates. The emulsifying properties of EWP were characterised in terms of droplet size, droplet charge (zeta potential), microstructure, phase separation and DH.

An experimental study was carried out initially to understand the effects of pH and heat treatment of EWL on the physical (turbidity) and electrical charge properties (zeta potential) and degree of denaturation of EWP. The results obtained indicated that regardless of the protein concentration of EWL, highest turbidity was observed at acidic pH (3, 4 and 5). This indicates that at acidic pH, protein aggregation and precipitation can occur leading to haziness or cloudiness in the EWL solution. With regards to changes in the electrical net charges of EWP, ζ -potential was not affected by protein concentration but was significantly changed by pH changes. Highest positive ζ -potential value was obtained at pH 2, while highest negative ζ -potential value was observed at pH 11 being increased gradually with increasing pH. At pH 5 close to the isoelectric point of most EWPs, ζ -potential was close to zero. The effect of heat treatment of EWL at various temperatures (57, 58, 59, 60 and 62°C) and at different times (0-19 minutes) was also investigated to determine the denaturation temperature of EWL. Higher turbidity and protein aggregation were observed as temperature increased from 57 to 62°C and when the heating time increased from 5 to 19 minutes. At 60°C, EWL began to thicken and after 5 minutes coagulation and gelation occurred rapidly. The results of pH and heat treatment have an important implication that protein aggregation and partial protein denaturation may be used to improve the emulsifying properties or other functional properties of EWP.

The investigations into the reduction of visible aggregates being formed when an emulsion is prepared with EWL or EWP solution has not been reported elsewhere. The effects of heat treatment and oil and protein concentrations on the formation of aggregates were studied. It was found that heat treatment (60°C for 30 minutes) of 1% w/w EWP solution prior to homogenisation did not have any effect on the reduction of aggregates in emulsions containing various oil concentrations. However, formation of aggregates was reduced significantly as oil concentration reduced to 5%, indicating that oil concentration played a significant factor causing the formation of aggregates and the formation of aggregates could be due to bridging flocculation. It was discovered that minimal to no aggregates could be produced either in emulsions containing 1% EWP and oil concentration of $\leq 6\%$ (w/w) or 3% EWP and 1% oil. The results obtained have provided important information that emulsions prepared from EWL can contain little or no aggregates which can help expand the applicability of EWP in various emulsion systems. However, in this study, the stability of emulsions over a long period of time was not investigated. Further stability studies need to be carried out to extend or improve its stability at the oil and protein concentrations mentioned above. Additionally, emulsions containing oil and protein concentrations used in this study also need to be prepared with egg white powder to compare the formation of aggregates or determine if aggregates will be formed using egg white powder like EWL.

Next, the effect of low EWP concentrations (0.1, 0.3, 0.5, 0.8, 1 and 2% w/w) on the formation and characteristics of 5% O/W emulsions was investigated. It was discovered that little or no aggregates was produced in emulsions containing 0.1-1% EWP. However, at 2% EWP aggregates formed were much larger. Droplet size was observed to increase significantly as protein concentration increased from 0.1 to 2%, in which smallest droplet size was observed at 0.3% and largest at 2% EWP concentration. Heat treatment of the emulsions was found to have no pronounced effect on emulsion oil droplet size and the emulsions produced showed no sign of instability. This suggests that EWP emulsions prepared with a protein concentration ranging from 0.1 to 2% were stable to heat treatment. The results also showed that the stability of emulsions was sensitive to the effect of NaCl and CaCl₂ salts. This was measured from an increase in droplet size and phase separation with increasing ionic strength. At higher protein concentration (0.8-2%), emulsions were however more stable to salt-induced flocculation possibly due to a multiple protective layer being formed around the emulsion droplet at high protein concentration. This implies that stability of emulsions prepared with EWP to salt-induced flocculation was dependent on

the EWP concentration. EWP-stabilised emulsions (1% EWP and 10% oil at pH 8.3) were analysed for their stability against pH changes. Extensive droplet aggregation was observed at pH 4 and 5 in 1% EWP-stabilised emulsions while no sign aggregation was observed at extremely acidic pH 2.0 and alkaline pH 9 and 10.

Lastly, the effects of enzyme type, enzyme concentration (E/S) and hydrolysis on the degree of hydrolysis (DH) and emulsifying properties of EWP hydrolysates were investigated. The results of DH showed that enzyme type and enzyme concentration significantly affected DH. Enzyme papain was found to yield the highest DH at 4% E/S after 120 minutes hydrolysis. On the other hand, ficin and bromelain yielded similar DH levels at 4% E/S after 120 minutes hydrolysis. The results also revealed that DH increased significantly with increasing enzyme concentration and hydrolysis time. The results of SDS-PAGE showed hydrolysis (digestion) of the major protein components of EWP such as ovalbumin and ovotransferrin, into smaller peptides.

Surprisingly, enzymatic hydrolysis was found to completely stop the formation of aggregates, which is observed to occur when preparing emulsions using EWL without enzyme hydrolysis. When compared to the control emulsions (no enzymatic hydrolysis of EWP), the emulsions prepared with 4% bromelain EWP hydrolysates (DH 5.16%) yielded smaller droplet sizes than the control emulsion regardless of the EWP concentration (1, 5 and 10% w/w) used. Droplet size was observed to increase with increasing EWP concentration in the control emulsions but decrease with increasing hydrolysed EWP concentration. However, phase separation was observed to occur in the emulsions prepared from enzymatically hydrolysed EWP immediately after homogenisation at all the protein concentrations used, while in the control emulsion, phase separation was seen only at 5% and 10% EWP. In emulsions (containing 1% EWP and 10% oil) prepared with ficin and bromelain (1% enzyme concentration; obtained after 2 or 4 hours) EWP hydrolysates, it was discovered that the ficin-induced EWP hydrolysates produced smaller droplet size than the bromelain hydrolysates. The emulsion droplet size of the 4 hours hydrolysates was smaller than those of the 2 hours hydrolysates for both ficin and bromelain. Phase separation was also observed in both emulsions prepared with ficin and bromelain hydrolysates at 1% enzyme concentrations the following day after preparation. Emulsions prepared with 4% bromelain hydrolysate (DH 5.16%) produced smaller droplet size than the hydrolysates of 1% bromelain (DH 4.10% and 4.87%). On the other hand, emulsions

prepared with 1% ficin hydrolysates (DH 4.03% and 4.96%) produced smaller droplet size than the 0.3% ficin hydrolysate (DH 3.01%). The results indicate that a higher DH of around 4 - 5% is required to produce emulsions with smaller droplets size when using ficin and bromelain hydrolysates. Further studies need to be carried out to investigate the effect of salt treatment, added stabilisers and hydrocolloids on the emulsifying and stability of EWP hydrolysates.

Overall the research project was successfully carried out and the research objectives were achieved. Nevertheless, to further commercialise the research outcomes of this research, further investigations may need to be carried out.

References

- Abbasnezhad, B., Hamdami, N., Monteau, J. Y., & Vatankhah, H. (2016). Numerical modelling of heat transfer and pasteurizing value during thermal processing of intact egg. *Food science & nutrition*, 4(1), 42-49.
- Abd El-Salam, M. H., & El-Shibiny, S. (2017). Preparation, properties, and uses of enzymatic milk protein hydrolysates. *Critical reviews in food science and nutrition*, 57(6), 1119-1132.
- Abeyrathne, E. D. N. S., Lee, H. Y., & Ahn, D. U. (2013). Egg white proteins and their potential use in food processing or as nutraceutical and pharmaceutical agents-A review. *Poultry Science*, 92(12), 3292-3299.
- Achouri, A., Zamani, Y., & Boye, J. I. (2012). Stability and physical properties of emulsions prepared with and without soy proteins. *Journal of Food Research*, 1(1), 254.
- Adjonu, R., Doran, G., Torley, P., & Agboola, S. (2014). Formation of whey protein isolate hydrolysate stabilised nanoemulsion. *Food Hydrocolloids*, 41, 169-177.
- Adler-Nissen, J. (1986). *Enzymic hydrolysis of food proteins*. Elsevier applied science publishers.
- Agboola, S. O., & Dalgleish, D. G. (1996). Enzymatic hydrolysis of milk proteins used for emulsion formation. 2. Effects of calcium, pH, and ethanol on the stability of the emulsions. *Journal of agricultural and food chemistry*, 44(11), 3637-3642.
- Agboola, S. O., Singh, H., Munro, P. A., Dalgleish, D. G., & Singh, A. M. (1998). Destabilization of oil-in-water emulsions formed using highly hydrolysed whey proteins. *Journal of Agricultural and Food Chemistry*, 46(1), 84-90.
- Aguilera, J. M., Stanley, D. W., & Barbosa-Cánovas, G. V. (1999). *Microstructural Principles of Food Processing Engineering*. Gaithersburg, MD: Aspen Publishers.
- Ahn, D. (2011). Egg components. *Animal Science Department. Iowa State University* <http://docs.google.com/viewer>.
- Akkouche, Z., Aissat, L., & Madani, K. (2012). Effect of heat on egg white proteins. In *International Conference on Applied Life Sciences*. InTech.
- Al-Hakkak, J., & Al-Hakkak, F. (2010). Functional egg white-pectin conjugates prepared by controlled Maillard reaction. *Journal of Food Engineering*, 100(1), 152-159.
- Alleoni, A. C. C. (2006). Albumen protein and functional properties of gelation and foaming. *Scientia Agricola*, 63(3), 291-298.
- Alizadeh-Pasdar, N., & Li-Chan, E. C. (2000). Comparison of protein surface hydrophobicity measured at various pH values using three different fluorescent probes. *Journal of Agricultural and Food Chemistry*, 48(2), 328-334.
- Altalhi, A. S. (2013). *Egg white foam* (Master's thesis). Massey University, Auckland New Zealand.
- Anton, M., Chapleau, N., Beaumal, V., Delepine, S., & de Lamballerie-Anton, M. (2001). Effect of high-pressure treatment on rheology of oil-in-water emulsions prepared with hen egg yolk. *Innovative Food Science & Emerging Technologies*, 2(1), 9-21.
- Aoki, T., Hiidome, Y., Kitahata, K., Sugimoto, Y., Ibrahim, H. R., & Kato, Y. (1999). Improvement of heat stability and emulsifying activity of ovalbumin by conjugation with glucuronic acid through the Maillard reaction. *Food Research International*, 32(2), 129-133.

- Amine, C., Dreher, J., Helgason, T., & Tadros, T. (2014). Investigation of emulsifying properties and emulsion stability of plant and milk proteins using interfacial tension and interfacial elasticity. *Food Hydrocolloids*, 39, 180-186.
- Arzeni, C., Pérez, O. E., & Pilosof, A. M. (2012). Functionality of egg white proteins as affected by high intensity ultrasound. *Food Hydrocolloids*, 29(2), 308-316.
- Azzolini, M., Tosi, E., Veneri, G., & Zapparoli, G. (2010). Evaluating the efficacy of lysozyme against lactic acid bacteria under different winemaking scenarios. *South African Journal Enology Viticulture*, 31, 99-105.
- Baron, F., Jan, S., & Jeantet, R. (2010). Qualité microbiologique des ovoproduits. *Science et technologie de l'oeuf*, 2, 321-349.
- Belitz, H. D., Grosch, W., & Schieberle, P. (2009). *Food Chemistry*. (4th Rev. ed.). Berlin, Germany: Springer.
- Bovšková, H., & Miková, K. (2011). Factors influencing egg white foam quality. *Czech Journal of Food Sciences*, 29(4), 322-327.
- Boyacı, D., Korel, F., & Yemenicioğlu, A. (2016). Development of activate-at-home-type edible antimicrobial films: An example pH-triggering mechanism formed for smoked salmon slices using lysozyme in whey protein films. *Food Hydrocolloids*, 60, 170-178.
- Brand, J., & Kulozik, U. (2016). Comparison of Different Mechanical Methods for the Modification of the Egg White Protein Ovomucin, Part B: Molecular Aspects. *Food and Bioprocess Technology*, 1-9.
- Caessens, P. W., Daamen, W. F., Gruppen, H., Visser, S., & Voragen, A. G. (1999). β -Lactoglobulin hydrolysis. 2. Peptide identification, SH/SS exchange, and functional properties of hydrolysate fractions formed by the action of plasmin. *Journal of agricultural and food chemistry*, 47(8), 2980-2990.
- Caivano, J. L., & del Pilar Buera, M. (Eds.). (2012). *Colour in food: technological and psychophysical aspects*. CRC Press.
- Campbell, N. F., Shih, F. F., & Marshall, W. E. (1992). Enzymic phosphorylation of soy protein isolate for improved functional properties. *Journal of Agricultural and Food Chemistry*, 40(3), 403-406.
- Campbell, L., Raikos, V., & Euston, S. R. (2003). Modification of functional properties of egg- white proteins. *Food/Nahrung*, 47(6), 369-376.
- Carballo, J., Barreto, G., & Colmenero, F. J. (1995). Starch and egg white influence on properties of bologna sausage as related to fat content. *Journal of Food Science*, 60(4), 673-677.
- Carvajal, P. A., MacDonald, G. A., & Lanier, T. C. (1999). Cryostabilization mechanism of fish muscle proteins by maltodextrins. *Cryobiology*, 38(1), 16-26.
- Cegielska-Radziejewska, R., Lesnierowski, G., & Kijowski, J. (2008). Properties and application of egg white lysozyme and its modified preparations-a review. *Polish Journal of Food and Nutrition Sciences*, 58(1).
- Chan, W. M., & Ma, C. Y. (1999). Acid modification of proteins from soymilk residue (okara). *Food Research International*, 32(2), 119-127.
- Chang, C., Niu, F., Su, Y., Qiu, Y., Gu, L., & Yang, Y. (2016). Characteristics and emulsifying properties of acid and acid-heat induced egg white protein. *Food Hydrocolloids*, 54, 342-350.

- Chang, C., Li, X., Li, J., Niu, F., Zhang, M., Zhou, B., ... & Yang, Y. (2017). Effect of enzymatic hydrolysis on characteristics and synergistic efficiency of pectin on emulsifying properties of egg white protein. *Food Hydrocolloids*, 65, 87-95.
- Chanamai, R. A. D. J. M., & McClements, D. J. (2002). Comparison of gum arabic, modified starch, and whey protein isolate as emulsifiers: influence of pH, CaCl₂ and temperature. *Journal of food science*, 67(1), 120-125.
- Charter, E. A., & Lagarde, G. (1999). Lysozyme and other proteins in eggs. *Encyclopaedia of Food Microbiology*, CA Batt, RK Robinson, P. Patel and PD Petel, Eds., Academic Press: Adelaide, 1582-1587.
- Chen, Y. C., Chang, H. S., Wang, C. T., & Cheng, F. Y. (2009). Antioxidative activities of hydrolysates from duck egg white using enzymatic hydrolysis. *Asian-Australasian Journal of Animal Sciences*, 22(11), 1587-1593.
- Chen, C., Chi, Y. J., Zhao, M. Y., & Lv, L. (2012). Purification and identification of antioxidant peptides from egg white protein hydrolysate. *Amino acids*, 43(1), 457-466.
- Chen, C., Chi, Y. J., Zhao, M. Y., & Xu, W. (2012). Influence of degree of hydrolysis on functional properties, antioxidant and ACE inhibitory activities of egg white protein hydrolysate. *Food Science and Biotechnology*, 21(1), 27-34.
- Cheng, Y., Xiong, Y. L., & Chen, J. (2010). Antioxidant and emulsifying properties of potato protein hydrolysate in soybean oil-in-water emulsions. *Food Chemistry*, 120(1), 101-108.
- Cho, D. Y., Jo, K., Cho, S. Y., Kim, J. M., Lim, K., Suh, H. J., & Oh, S. (2014). Antioxidant effect and functional properties of hydrolysates derived from egg-white protein. *Korean Journal for Food Science of Animal Resources*, 34(3), 362.
- Chobert, J. M., Bertrand-Harb, C., & Nicolas, M. G. (1988). Solubility and emulsifying properties of caseins and whey proteins modified enzymically by trypsin. *Journal of Agricultural and Food Chemistry*, 36(5), 883-892.
- Choi, S. J., E. A. Decker, L. Henson, L. M. Popplewell, & D. J. McClements. (2010). Influence of droplet charge on the chemical stability of citral in oil-in-water emulsions. *Journal of Food Science*, 75(6) C536– C540.
- Chu, B., Ichikawa, S., Kanafusa, S. & Nakajima, M. (2008). Stability of protein-stabilized β -carotene nanodispersions against heating, salts and pH. *Journal of the Science of Food and Agriculture*, 88(10), 1764-1769.
- Chung, C., Sher, A., Rousset, P., Decker, E. A., & McClements, D. J. (2017). Formulation of food emulsions using natural emulsifiers: Utilization of quillaja saponin and soy lecithin to fabricate liquid coffee whiteners. *Journal of Food Engineering*, 209, 1-11.
- Church, F. C., Swaisgood, H. E., Porter, D. H., & Catignani, G. L. (1983). Spectrophotometric assay using o-phthalaldehyde for determination of proteolysis in milk and isolated milk proteins1. *Journal of Dairy Science*, 66(6), 1219-1227.
- Clemente, A. (2000). Enzymatic protein hydrolysates in human nutrition. *Trends in Food Science & Technology*, 11(7), 254-262.
- Clydesdale, F. M. & F. J. Francis. (1975). *Food Colorimetry: Theory and Applications*. Westport, CT: AVI Publishing.
- Conde, J. M., & Patino, J. M. R. (2007). The effect of enzymatic treatment of a sunflower protein isolate on the rate of adsorption at the air–water interface. *Journal of Food Engineering*, 78(3), 1001-1009.

- Cotterill, O. J., Gardner, F. A., Cunningham, F. E., & Funk, E. M. (1959). Titration curves and turbidity of whole egg white. *Poultry Science*, 38(4), 836-842.
- Croguennec, T., Nau, F., & Brule, G. (2002). Influence of pH and salts on egg white gelation. *Journal of Food Science*, 67(2), 608-614.
- Croguennec, T., Renault, A., Beauflis, S., Dubois, J. J., & Pezennec, S. (2007). Interfacial properties of heat-treated ovalbumin. *Journal of colloid and interface science*, 315(2), 627-636.
- Cunningham, F. E. (1995). Egg product pasteurization. In: W. J. Stadelman & O. J. (Eds.), *Cotterill Egg Science and Technology* (pp. 289-321). Binghamton, NY: Haworth Press.
- Daeschel, M. A., Bruslind, L., & Clawson, J. (1999). Application of the enzyme lysozyme in brewing. *Technical Quarterly-Master Brewers Association of The Americas*, 36, 219-222.
- Dagorn- Scaviner, C., Gueguen, J., & Lefebvre, J. (1987). Emulsifying properties of pea globulins as related to their adsorption behaviours. *Journal of Food Science*, 52(2), 335-341.
- Dalgleish, D. G. (1997). Adsorption of protein and the stability of emulsions. *Trends in food science & technology*, 8(1), 1-6.
- Damodaran, S. (2005). Protein stabilization of emulsions and foams. *Journal of Food Science*, 70(3).
- Damodaran, S., Parkin, K. L., & Fennema, O. R. (2008). *Fennema's Food Chemistry* (4th ed.). Boca Raton: CRC Press.
- Davalos, A., Miguel, M., Bartolome, B., & Lopez-Fandino, R. (2004). Antioxidant activity of peptides derived from egg white proteins by enzymatic hydrolysis. *Journal of Food Protection*, 67(9), 1939-1944.
- Degner, B. M., Chung, C., Schlegel, V., Hutkins, R., & McClements, D. J. (2014). Factors Influencing the Freeze- Thaw Stability of Emulsion- Based Foods. *Comprehensive Reviews in Food Science and Food Safety*, 13(2), 98-113.
- Del Nobile, M. A., Conte, A., Buonocore, G. G., Incoronato, A. L., Massaro, A., & Panza, O. (2009). Active packaging by extrusion processing of recyclable and biodegradable polymers. *Journal of Food Engineering*, 93(1), 1-6.
- Delahaije, R. J., Wierenga, P. A., van Nieuwenhuijzen, N. H., Giuseppin, M. L., & Gruppen, H. (2013). Protein concentration and protein-exposed hydrophobicity as dominant parameters determining the flocculation of protein-stabilized oil in-water emulsions. *Langmuir*, 29(37), 11567-11574.
- Delahaije, R. J., Gruppen, H., Giuseppin, M. L., & Wierenga, P. A. (2015). Towards predicting the stability of protein-stabilized emulsions. *Advances in colloid and interface science*, 219, 1-9.
- Desfougères, Y., Lechevalier, V., Pezennec, S., Artzner, F., & Nau, F. (2008). Dry-heating makes hen egg white lysozyme an efficient foaming agent and enables its bulk aggregation. *Journal of agricultural and food chemistry*, 56(13), 5120-5128.
- Dickinson, E., & Galazka, V. B. (1991). Bridging flocculation induced by competitive adsorption: implications for emulsion stability. *Journal of the Chemical Society, Faraday Transactions*, 87(7), 963-969.
- Dickinson, E. (1992). *An Introduction to Food Colloids*. Oxford: New York: Oxford University Press.

- Dickinson, E. (1994). Emulsions and droplet size control. In *Controlled particle, droplet and bubble formation* (pp. 189-216). Butterworth-Heinemann, Oxford.
- Dickinson, E., & Golding, M. (1997). Rheology of sodium caseinate stabilized oil-in-water emulsions. *Journal of Colloid and Interface Science*, 191(1), 166-176.
- Dickinson, E., & Golding, M. (1998). Influence of calcium ions on creaming and rheology of emulsions containing sodium caseinate. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 144(1), 167-177.
- Dickinson, E. (2003). Hydrocolloids at interfaces and the influence on the properties of dispersed systems. *Food Hydrocolloids*, 17(1), 25-39.
- Dickinson, E. (2009). Hydrocolloids as emulsifiers and emulsion stabilizers. *Food hydrocolloids*, 23(6), 1473-1482.
- Dickinson, E. (2010). Flocculation of protein-stabilized oil-in-water emulsions. *Colloids and Surfaces B: Biointerfaces*, 81(1), 130-140.
- Dickinson, E. (2012). Emulsion gels: The structuring of soft solids with protein-stabilized oil droplets. *Food Hydrocolloids*, 28(1), 224-241.
- Dłużewska, E., Stabiecka, A., & Maszewska, M. (2006). Effect of oil phase concentration on rheological properties and stability of beverage emulsion. *Acta Scientiarum Polonorum Technologia Alimentaria*, 5(2), 147-156.
- Doi, E., Tani, F., Murata, M., Koseki, I., & Kitabatake, N. (1993). In Nishinari, K. and Doi, E. *Food Hydrocolloids: Structure, Properties and Functions*, 317-326.
- Drakos, A., & Kiosseoglou, V. (2006). Stability of acidic egg white protein emulsions containing xanthan gum. *Journal of Agricultural and Food Chemistry*, 54(26), 10164-10169.
- Drapala, K. P., Auty, M. A., Mulvihill, D. M., & O'Mahony, J. A. (2016). Improving thermal stability of hydrolysed whey protein-based infant formula emulsions by protein-carbohydrate conjugation. *Food Research International*, 88, 42-51.
- Dubey, R. R., & Parikh, R. H. (2004). Two-stage optimization process for formulation of chitosan microspheres. *AAPS Pharm Sci Tech*, 5(1), 20-28.
- Dudkiewicz, A., K. Tiede, K. Loeschner, L. H. S. Jensen, E. Jensen, R. Wierzbicki, A. B. A. Boxall, and K. Molhave (2011). Characterization of nanomaterials in food by electron microscopy. *TrAC Trends in Analytical Chemistry* 30(1), 28-43.
- Dybowska, B. E. (2008). Properties of milk protein concentrate stabilized oil-in-water emulsions. *Journal of food engineering*, 88(4), 507-513.
- Eleya, M. M., & Gunasekaran, S. (2002). Gelling Properties of Egg White Produced Using a Conventional and a Low- shear Reverse Osmosis Process. *Journal of food science*, 67(2), 725-729.
- Egerton, R. (2008). *Physical Principles of Electron Microscopy: An Introduction to TEM, SEM, and AEM*. New York: Springer Science.
- Enomoto, H., Li, C. P., Morizane, K., Ibrahim, H. R., Sugimoto, Y., Ohki, S., ... & Aoki, T. (2008). Improvement of Functional Properties of Bovine Serum Albumin through Phosphorylation by Dry- Heating in the Presence of Pyrophosphate. *Journal of food science*, 73(2).
- Erçelebi, E. A., & Ibanoglu, E. (2009). Rheological properties of whey protein isolate stabilized emulsions with pectin and guar gum. *European Food Research and Technology*, 229(2), 281-286.

- Erol, K., Köse, K., Uzun, L., Say, R., & Denizli, A. (2016). Polyethyleneimine assisted-two-step polymerization to develop surface imprinted cryogels for lysozyme purification. *Colloids and Surfaces B: Biointerfaces*, 146, 567-576.
- Ettoumi, Y. L., Chibane, M., & Romero, A. (2016). Emulsifying properties of legume proteins at acidic conditions: Effect of protein concentration and ionic strength. *LWT-Food Science and Technology*, 66, 260-266.
- Euston, S. R., Finnigan, S. R., & Hirst, R. L. (2000). Aggregation kinetics of heated whey protein-stabilized emulsions. *Food Hydrocolloids*, 14(2), 155-161.
- Euston, S. R., S. R. Finnigan, & R. L. Hirst. (2001). Heat-induced destabilization of oil-in-water emulsions formed from hydrolysed whey protein. *Journal of Agricultural and Food Chemistry*, 49(11) 5576– 5583.
- Fisicaro, E., Compari, C., & Braibanti, A. (2011). Hydrophobic hydration processes: Thermal and chemical denaturation of proteins. *Biophysical Chemistry*, 156(1), 51-67.
- Floury, J., J. Legrand, & A. Desrumaux. (2004). Analysis of a new type of high pressure homogeniser. Part B. Study of droplet break-up and recoalescence phenomena. *Chemical Engineering Science*, 59(6) 1285– 1294.
- Foegeding, E. A., & Davis, J. P. (2011). Food protein functionality: a comprehensive approach. *Food Hydrocolloid* .25(8),1853–1864.
- Friberg, S., Larsson, K., & Sjoblom, J. (2004). *Food Emulsions*. Marcel Dekker, New York.
- Galazka, V. B., Dickinson, E., & Ledward, D. A. (2000). Emulsifying properties of ovalbumin in mixtures with sulphated polysaccharides: effects of pH, ionic strength, heat and high- pressure treatment. *Journal of the Science of Food and Agriculture*, 80(8), 1219-1229.
- Gao, Z., Zhao, J., Huang, Y., Yao, X., Zhang, K., Fang, Y., ... & Yang, H. (2017). Edible Pickering emulsion stabilized by protein fibrils. Part 1: Effects of pH and fibrils concentration. *LWT-Food Science and Technology*, 76, 1-8.
- Garcés-Rimón, M., Sandoval, M., Molina, E., López-Fandiño, R., & Miguel, M. (2016). Egg protein hydrolysates: New culinary textures. *International Journal of Gastronomy and Food Science*, 3, 17-22.
- Gharsallaoui, A., Cases, E., Chambin, O., & Saurel, R. (2009). Interfacial and emulsifying characteristics of acid-treated pea protein. *Food Biophysics*, 4(4), 273-280.
- Gauthier, S. F., Paquin, P., Pouliot, Y., & Turgeon, S. (1993). Surface Activity and Related Functional Properties of Peptides Obtained from Whey Proteins1. *Journal of Dairy Science*, 76(1), 321-328.
- Ghosh, S., & Coupland, J. N. (2008). Factors affecting the freeze–thaw stability of emulsions. *Food Hydrocolloids*, 22(1), 105-111.
- Gill, A. O., & Holley, R. A. (2000). Inhibition of bacterial growth on ham and bologna by lysozyme, nisin and EDTA. *Food Research International*, 33(2), 83-90.
- GoTo, Y., Calciano, L. J., & Fink, A. L. (1990). Acid-induced folding of proteins. *Proceedings of the National Academy of Sciences*, 87(2), 573-577.
- Greyling, N. (2017). *Optimisation of enzymatic hydrolysis of monkfish heads for preparing protein hydrolysates as animal feed ingredient* (Doctoral dissertation, Stellenbosch: Stellenbosch University).
- Gu, Y. S., Decker, E. A., & McClements, D. J. (2005). Influence of pH and carrageenan type on properties of β -lactoglobulin stabilized oil-in-water emulsions. *Food Hydrocolloids*, 19(1), 83-91.

- Güçbilmez, Ç. M., Yemenicioğlu, A., & Arslanoğlu, A. (2007). Antimicrobial and antioxidant activity of edible zein films incorporated with lysozyme, albumin proteins and disodium EDTA. *Food research international*, 40(1), 80-91.
- Guo, Q., & Mu, T. H. (2011). Emulsifying properties of sweet potato protein: effect of protein concentration and oil volume fraction. *Food Hydrocolloids*, 25(1), 98-106.
- Guzzo, F., Cappello, M. S., Azzolini, M., Tosi, E., & Zapparoli, G. (2011). The inhibitory effects of wine phenolics on lysozyme activity against lactic acid bacteria. *International journal of food microbiology*, 148(3), 184-190.
- Hagolle, N., Relkin, P., Popineau, Y., & Bertrand, D. (2000). Study of the stability of egg white protein- based foams: effect of heating protein solution. *Journal of the Science of Food and Agriculture*, 80(8), 1245-1252.
- Hammershoj, M., Nebel, C., & Carstens, J. H. (2008). Enzymatic hydrolysis of ovomucin and effect on foaming properties. *Food Res. Int.* 41, 522–531.
- Hartel, R. W. (2001). *Crystallization in foods*. Aspen Publishers.
- Hasenhuettl, G. L. (2008). Synthesis and commercial preparation of food emulsifiers. In G. L. Hasenhuettl & R. W. Hartel (Eds.), *Food emulsifiers and their applications* (pp. 11-37). New York: Springer.
- Hatta, H., Hagi, T., & Hirano, K. (1997). Chemical and physicochemical properties of hen eggs and their application in foods. *Hen Eggs: Basic and Applied Science*, 117.
- Hayashi, Y., Nagano, S., Enomoto, H., Li, C. P., Sugimoto, Y., Ibrahim, H. R., ... & Aoki, T. (2009). Improvement of Foaming Property of Egg White Protein by Phosphorylation through Dry- Heating in the Presence of Pyrophosphate. *Journal of food science*, 74(1).
- Hammershøj, M., Rasmussen, H. C., Carstens, J. H., & Pedersen, H. (2006). Dry-pasteurization of egg albumen powder in a fluidized bed. II. Effect on functional properties: gelation and foaming. *International journal of food science & technology*, 41(3), 263-274.
- Hebishy, E., Buffa, M., Guamis, B., Blasco-Moreno, A., & Trujillo, A. J. (2015). Physical and oxidative stability of whey protein oil-in-water emulsions produced by conventional and ultra high-pressure homogenization: Effects of pressure and protein concentration on emulsion characteristics. *Innovative Food Science & Emerging Technologies*, 32, 79-90.
- Hemar, Y., Tamehana, M., Munro, P. A., & Singh, H. (2001). Influence of xanthan gum on the formation and stability of sodium caseinate oil-in-water emulsions. *Food hydrocolloids*, 15(4), 513-519.
- Herpandi, N. H., Rosma, A., & Wan Nadiyah, W. A. (2011). The tuna fishing industry: A new outlook on fish protein hydrolysates. *Comprehensive Reviews in Food Science and Food Safety*, 10(4), 195-207.
- Hiidenhovi, J. (2007). Ovomucin. In *Bioactive Egg Compounds* (pp. 61-68). Springer Berlin Heidelberg.
- Hillgren, A., Lindgren, J., & Aldén, M. (2002). Protection mechanism of Tween 80 during freeze–thawing of a model protein, LDH. *International Journal of Pharmaceutics*, 237(1), 57-69.
- Horax, R., Vallecios, M. S., Hettiarachchy, N., Osorio, L. F., & Chen, P. (2017). Solubility, functional properties, ACE- I inhibitory and DPPH scavenging activities of Alcalase hydrolysed soy protein hydrolysates. *International Journal of Food Science & Technology*, 52(1), 196-204.

- Horne, D. S. (2011). Analytical Methods Light Scattering Techniques. *Encyclopaedia of Dairy Sciences*. 2nd ed. Academic Press.
- Horvath, H. (2009). Gustav Mie ad the scattering and adsorption of light by particles: Historic developments and basics. *Journal of Quantitative Spectroscopy and Radiative Transfer*, 10(11), 787-799.
- Hu, M., McClements, D. J., & Decker, E. A. (2003). Lipid oxidation in corn oil-in-water emulsions stabilized by casein, whey protein isolate, and soy protein isolate. *Journal of Agricultural and Food Chemistry*, 51(6), 1696-1700.
- Huang, W. Y., Majumder, K., & Wu, J. (2010). Oxygen radical absorbance capacity of peptides from egg white protein ovotransferrin and their interaction with phytochemicals. *Food Chemistry*, 123(3), 635-641.
- Huang, P. H., Lu, H. T., Wang, Y. T., & Wu, M. C. (2011). Antioxidant activity and emulsion-stabilizing effect of pectic enzyme treated pectin in soy protein isolate-stabilized oil/water emulsion. *Journal of agricultural and food chemistry*, 59(17), 9623-9628.
- Huang, Q., Qui, N., Ma, M. H., Jin, Y. G., Yang, H., Geng, F., & Sun, S. H. (2012). Estimation of egg freshness using S-ovalbumin as an indicator. *Poultry Science*, 91,739–743.
- Hui, Y. H., & Al-Holy, M. A. (Eds.). (2007). Food chemistry: Principles and Applications (2nd ed.). West Sacramento, CA: Science Technology System.
- Huopalahti, R., Lopez-Fandino, R., Anton, M., & Schade, R. (Ed.). (2007). Bioactive Egg Compounds. Berlin London: Springer.
- Hunt, J. A., & Dalgleish, D. G. (1995). Heat Stability of Oil- in- Water Emulsions Containing Milk Proteins: Effect of Ionic Strength and pH. *Journal of food science*, 60(5), 1120-1123.
- Hunter, R. J. (1986). *Foundations of colloid science Volume 1*. Oxford: Oxford University Press.
- Huntington, J. A., & Stein, P. E. (2001). Structure and properties of ovalbumin. *Journal of Chromatography B: Biomedical Sciences and Applications*, 756(1), 189-198.
- Hutchings, J. B. (1999). *Food Colour and Appearance*. New York: Springer.
- Ikeda, S., & Morris, V. J. (2002). Fine-stranded and particulate aggregates oMf heat-denatured whey proteins visualized by atomic force microscopy. *Biomacromolecules*, 3(2), 382-389.
- Jamdar, S. N., Rajalakshmi, V., Pednekar, M. D., Juan, F., Yardi, V., & Sharma, A. (2010). Influence of degree of hydrolysis on functional properties, antioxidant activity and ACE inhibitory activity of peanut protein hydrolysate. *Food Chemistry*, 121(1), 178-184.
- Jafari, S. M., He, Y., & Bhandari, B. (2006). Nano-emulsion production by sonication and microfluidization—a comparison. *International Journal of Food Properties*, 9(3), 475-485.
- Jafari, S. M., Beheshti, P., & Assadpoor, E. (2012). Rheological behaviour and stability of D-limonene emulsions made by a novel hydrocolloid (Angum gum) compared with Arabic gum. *Journal of Food Engineering*, 109(1), 1-8.
- Jiang, J., Chen, J., & Xiong, Y. L. (2009). Structural and emulsifying properties of soy protein isolate subjected to acid and alkaline pH-shifting processes. *Journal of Agricultural and Food Chemistry*, 57(16), 7576-7583.

- Johnson, H. S., & Ridlen, S. F. (2016). Structure of the Egg. In Incubation and Embryology. University of Illinois Extension.
- Joshi, M., Adhikari, B., Aldred, P., Panozzo, J. F., Kasapis, S., & Barrow, C. J. (2012). Interfacial and emulsifying properties of lentil protein isolate. *Food chemistry*, 134(3), 1343-1353.
- Joye, I. J., & McClements, D. J. (2014). Biopolymer-based nanoparticles and microparticles: fabrication, characterization, and application. *Current Opinion in Colloid & Interface Science*, 19(5), 417-427.
- Juneja, V. K., Dwivedi, H. P., & Yan, X. (2012). Novel natural food antimicrobials. *Annual review of food science and technology*, 3, 381-403.
- Jung, S., Murphy, P. A., & Johnson, L. A. (2005). Physicochemical and functional properties of soy protein substrates modified by low levels of protease hydrolysis. *Journal of Food Science*, 70(2), 180-187.
- Kaewmanee, T., Benjakul, S., & Visessanguan, W. (2011). Effect of NaCl on thermal aggregation of egg white proteins from duck egg. *Food chemistry*, 125(2), 706-712.
- Kato, A., & Nakai, S. (1980). Hydrophobicity determined by a fluorescence probe method and its correlation with surface properties of proteins. *Biochimica et biophysica acta (BBA)-Protein structure*, 624(1), 13-20.
- Kato, A., Osako, Y., Matsudomi, N., & Kobayashi, K. (1983). Changes in the emulsifying and foaming properties of proteins during heat denaturation. *Agricultural and Biological Chemistry*, 47(1), 33-37.
- Kato, A., Ibrahim, H. R., Watanabe, H., Honma, K., & Kobayashi, K. (1989). New approach to improve the gelling and surface functional properties of dried egg white by heating in dry state. *Journal of Agricultural and Food Chemistry*, 37(2), 433-437.
- Kato, A., Minaki, K., & Kobayashi, K. (1993). Improvement of emulsifying properties of egg white proteins by the attachment of polysaccharide through Maillard reaction in a dry state. *Journal of Agricultural and Food Chemistry*, 41(4), 540-543.
- Kavanagh, G. M., Clark, A. H., & Ross-Murphy, S. B. (2000). Heat-induced gelation of globular proteins: part 3. Molecular studies on low pH β -lactoglobulin gels. *International Journal of Biological Macromolecules*, 28(1), 41-50.
- Keowmaneechai, E., & McClements, D. J. (2006). Influence of EDTA and citrate on thermal stability of whey protein stabilized oil-in-water emulsions containing calcium chloride. *Food research international*, 39(2), 230-239.
- Keowmaneechai, E., & McClements, D. J. (2002). Influence of EDTA and citrate on physicochemical properties of whey protein-stabilized oil-in-water emulsions containing CaCl₂. *Journal of Agricultural and Food Chemistry*, 50(24), 7145-7153.
- Kim, H. J., Decker, E. A., & McClements, D. J. (2002). Role of postadsorption conformation changes of β -lactoglobulin on its ability to stabilize oil droplets against flocculation during heating at neutral pH. *Langmuir*, 18(20), 7577-7583.
- Kim, H., Decker, E. A., & McClements, D. J. (2005). Influence of protein concentration and order of addition on thermal stability of β -lactoglobulin stabilized n-hexadecane oil-in-water emulsions at neutral pH. *Langmuir*, 21(1), 134-139.
- Kitabatake, N., Indo, K., & Doi, E. (1989). Changes in interfacial properties of hen egg ovalbumin caused by freeze-drying and spray-drying. *Journal of Agricultural and Food Chemistry*, 37(4), 905-910.

- Klang, V., N. B. Matsko, C. Valenta, and F. Hofer (2012). Electron microscopy of nanoemulsions: An essential tool for characterisation and stability assessment. *Micron* 43 (2–3), 85-103.
- Klompong, V., Benjakul, S., Kantachote, D., & Shahidi, F. (2007). Antioxidative activity and functional properties of protein hydrolysate of yellow stripe trevally (*Selaroides leptocephalus*) as influenced by the degree of hydrolysis and enzyme type. *Food chemistry*, 102(4), 1317-1327.
- Kong, X., Zhou, H., & Qian, H. (2007). Enzymatic preparation and functional properties of wheat gluten hydrolysates. *Food Chemistry*, 101(20), 615-620.
- Kristinsson, H. G., & Rasco, B. A. (2000). Fish protein hydrolysates: production, biochemical, and functional properties. *Critical reviews in food science and nutrition*, 40(1), 43-81.
- Kudryashova, E. V., Visser, A. J., van Hoek, A., & de Jongh, H. H. (2007). Molecular details of ovalbumin–pectin complexes at the air/water interface: A spectroscopic study. *Langmuir*, 23(15), 7942-7950.
- Kulmyrzaev, A., Bryant, C., & McClements, D. J. (2000). Influence of sucrose on the thermal denaturation, gelation, and emulsion stabilization of whey proteins. *Journal of Agricultural and Food Chemistry*, 48(5), 1593-1597.
- Kulmyrzaev, A. A., & Schubert, H. (2004). Influence of KCl on the physicochemical properties of whey protein stabilized emulsions. *Food Hydrocolloids*, 18(1), 13-19.
- Lam, R. S., & Nickerson, M. T. (2013). Food proteins: a review on their emulsifying properties using a structure–function approach. *Food chemistry*, 141(2), 975-984.
- Lamsal, B. P., Jung, S., & Johnson, L. A. (2007). Rheological properties of soy protein hydrolysates obtained from limited enzymatic hydrolysis. *LWT-Food Science and Technology*, 40(7), 1215-1223.
- Lechevalier, V., Jeantet, R., Arhaliass, A., Legrand, J., & Nau, F. (2007). Egg white drying: Influence of industrial processing steps on protein structure and functionalities. *Journal of Food Engineering*, 83(3), 404-413.
- Lechevalier, V., Guérin-Dubiard, C., Anton, M., Beaumal, V., Briand, E. D., Gillard, A., ... & Nau, F. (2017a). Effect of dry heat treatment of egg white powder on its functional, nutritional and allergenic properties. *Journal of Food Engineering*, 195, 40-51.
- Lechevalier, V., Guérin-Dubiard, C., Anton, M., Beaumal, V., Briand, E. D., Gillard, A., ... & Dupont, D. (2017b). Pasteurisation of liquid whole egg: Optimal heat treatments in relation to its functional, nutritional and allergenic properties. *Journal of Food Engineering*, 195, 137-149.
- Lee, S. W., Shimizu, M., Kaminogawa, S., & Yamauchi, K. (1987). Emulsifying properties of peptides obtained from the hydrolysates of β -casein. *Agricultural and biological chemistry*, 51(1), 161-166.
- Li, C. P., Ibrahim, H. R., Sugimoto, Y., Hatta, H., & Aoki, T. (2004). Improvement of functional properties of egg white protein through phosphorylation by dry-heating in the presence of pyrophosphate. *Journal of agricultural and food chemistry*, 52(18), 5752-5758.
- Li, L. C., & Tian, Y. (2007). Zeta potential. *Encyclopedia of Pharmaceutical Technology*, 4117-4128 ([10.1081/E-EPT-100200015](https://doi.org/10.1081/E-EPT-100200015)).
- Liang, Y., Patel, H., Matia-Merino, L., Ye, A., & Golding, M. (2013). Structure and stability of heat-treated concentrated dairy-protein-stabilised oil-in-water

- emulsions: A stability map characterisation approach. *Food hydrocolloids*, 33(2), 297-308.
- Liang, Y., Wong, S. S., Pham, S. Q., & Tan, J. J. (2016). Effects of globular protein type and concentration on the physical properties and flow behaviours of oil-in-water emulsions stabilized by micellar casein–globular protein mixtures. *Food Hydrocolloids*, 54, 89-98.
- Liang, Y., Matia-Merino, L., Gillies, G., Patel, H., Ye, A., & Golding, M. (2017). The Heat Stability of Milk Protein-stabilized Oil-in-water Emulsions: A Review. *Current Opinion in Colloid & Interface Science*.
- Liburdi, K., Benucci, I., & Esti, M. (2014). Lysozyme in wine: an overview of current and future applications. *Comprehensive Reviews in Food Science and Food Safety*, 13(5), 1062-1073.
- Li-Chan, E., & Nakai, S. (1989). Biochemical basis for the properties of egg white. *Critical Reviews in Poultry Biology*, 2, 21-57.
- Li-Chan, E. C., Powrie, W. D., & Nakai, S. (1995). The chemistry of eggs and egg products. *Egg science and technology*, 4, 105-75.
- Li-Chan, E. C., & Kim, H. O. (2008). Structure and chemical composition of eggs. *Egg bioscience and biotechnology*, 1-96.
- Liu, Q., Kong, B., Xiong, Y. L., & Xia, X. (2010). Antioxidant activity and functional properties of porcine plasma protein hydrolysate as influenced by the degree of hydrolysis. *Food Chemistry*, 118(2), 403-410.
- Lizarraga, M. S., Pan, L. G., Anon, M. C., & Santiago, L. G. (2008). Stability of concentrated emulsions measured by optical and rheological methods. Effect of processing conditions—I. Whey protein concentrate. *Food Hydrocolloids*, 22(5), 868-878.
- Lomakina, K., & Mikova, K. (2006). A study of the factors affecting the foaming properties of egg white—a review. *Czech J. Food Sci*, 24(3), 110-118.
- Lopez, I., Santamaria, P., Tenorio, C., Garijo, P., Gutierrez, A. R., & Lopez, R. (2009). Evaluation of lysozyme to control vinification process and histamine production in Rioja wines. *Journal of Microbiology and Biotechnology*, 19(9), 1005-1012.
- López-Pedemonte, T. J., Roig-Sagués, A. X., Trujillo, A. J., Capellas, M., & Guamis, B. (2003). Inactivation of spores of *Bacillus cereus* in cheese by high hydrostatic pressure with the addition of nisin or lysozyme. *Journal of Dairy Science*, 86(10), 3075-3081.
- Lv, L., & Chi, Y. (2012). Improvement of functional properties of ovalbumin phosphorylated by dry-heating in the presence of pyrophosphate. *European Food Research and Technology*, 235(5), 981-987.
- Machado, F. F., Coimbra, J. S., Rojas, E. E. G., Minim, L. A., Oliveira, F. C., & Rita de Cássia, S. S. (2007). Solubility and density of egg white proteins: Effect of pH and saline concentration. *LWT-food Science and Technology*, 40(7), 1304-1307.
- Makki, F., & Durance, T. D. (1996). Thermal inactivation of lysozyme as influenced by pH, sucrose and sodium chloride and inactivation and preservative effect in beer. *Food research international*, 29(7), 635-645.
- Malvern Instruments Limited. (2014). Zeta Potential. An Introduction in 30 Minutes. Retrieved from <http://www3.nd.edu/~rroeder/ame60647/slides/zeta.pdf>.
- Manso, M. A., Miguel, M., Even, J., Hernández, R., Aleixandre, A., & López-Fandiño, R. (2008). Effect of the long-term intake of an egg white hydrolysate on the oxidative

- status and blood lipid profile of spontaneously hypertensive rats. *Food chemistry*, 109(2), 361-367.
- Manzoor, Z., Nawaz, A., Mukhtar, H., & Haq, I. (2016). Bromelain: Methods of Extraction, Purification and Therapeutic Applications. *Brazilian Archives of Biology and Technology*, 59.
- Memarpoor-Yazdi, M., Asoodeh, A., & Chamani, J. (2012). A novel antioxidant and antimicrobial peptide from hen egg white lysozyme hydrolysates. *Journal of Functional Foods*, 4(1), 278-286.
- McClements, D. J. (1999). Emulsion formation. *Food emulsions. Principles, practice and techniques*. CRC Press, Boca Raton, 161-184.
- McClements, D. J., & Decker, E. A. (2000). Lipid oxidation in oil- in- water emulsions: Impact of molecular environment on chemical reactions in heterogeneous food systems. *Journal of Food Science*, 65(8), 1270-1282.
- McClements, D. J. (2000). Comments on viscosity enhancement and depletion flocculation by polysaccharides. *Food Hydrocolloids*, 14(2), 173-177.
- McClements, D. J. (2002). Modulation of globular protein functionality by weakly interacting cosolvents. *Critical reviews in food science and nutrition*, 42(5), 417-471.
- McClements, D. J. (2004). Protein-stabilized emulsions. *Current Opinion in Colloid & Interface Science*, 9(5), 305-313.
- McClements, D. J. (2005). *Food emulsions: principles, practices, and techniques* (3rd ed.). Boca Raton, Florida: CRC Press.
- McClements, D. J. (2007). Critical review of techniques and methodologies for characterization of emulsion stability. *Critical Reviews in Food Science and Nutrition*, 47(7) 611– 649.
- McClements, D. J. & J. Rao. (2011). Food-grade nanoemulsions: Formulation, fabrication, properties, performance, biological fate, and potential toxicity. *Critical Reviews in Food Science and Nutrition*, 51(4) 285– 330.
- McClements, D. J., L. Henson, L. M. Popplewell, E. A. Decker, & S. J. Choi. (2012). Inhibition of ostwald ripening in model beverage emulsions by addition of poorly water-soluble triglyceride oils. *Journal of Food Science*, 77(1) C33– C38.
- McClements, D. J. (2015). *Food emulsions: principles, practices, and techniques*, third edition. Retrieved from <http://ebookcentral.proquest.com>.
- McSweeney, S. L., Mulvihill, D. M., & O'Callaghan, D. M. (2004). The influence of pH on the heat-induced aggregation of model milk protein ingredient systems and model infant formula emulsions stabilized by milk protein ingredients. *Food Hydrocolloids*, 18(1), 109-125.
- Miedzianka, J., & Pęksa, A. (2013). Effect of pH on phosphorylation of potato protein isolate. *Food chemistry*, 138(4), 2321-2326.
- Miguel, M., Alonso, M. J., Salaices, M., Aleixandre, A., & López-Fandiño, R. (2007). Antihypertensive, ACE-inhibitory and vasodilator properties of an egg white hydrolysate: Effect of a simulated intestinal digestion. *Food Chemistry*, 104(1), 163-168.
- Mine, Y., Noutomi, T., & Haga, N. (1990). Thermally induced changes in egg white proteins. *Journal of Agricultural Food Chemistry*, 38(12), 2122-2125.
- Mine, Y., Noutomi, T., & Haga, N. (1991). Emulsifying and structural properties of ovalbumin. *Journal of Agricultural and Food Chemistry*, 39(3), 443-446.

- Mine, Y. (1995). Recent advances in the understanding of egg white protein functionality. *Trends in Food Science & Technology*, 6(7), 225-232.
- Mine, Y. (1997). Effect of dry heat and mild alkaline treatment on functional properties of egg white proteins. *Journal of Agricultural and Food Chemistry*, 45(8), 2924-2928.
- Mine, Y. (2002). Recent advances in egg protein functionality in the food system. *World's Poultry Science Journal*, 58(1), 31-39.
- Mine, Y., & Yang, M. (2007). Concepts of hypoallergenicity. *Bioactive egg compounds*, 145-158.
- Mine, Y., & Yang, M. (2008). Recent advances in the understanding of egg allergens: basic, industrial, and clinical perspectives. *Journal of agricultural and food chemistry*, 56(13), 4874-4900.
- Molina-Bolivar, J. A., Galisteo-González, F., & Hidalgo-Alvarez, R. (2001). Specific adsorption on protein-covered particles and its influence on colloidal stability. *Colloids and Surfaces B: Biointerfaces*, 21(1), 125-135.
- Monfort, S., Mañas, P., Condón, S., Raso, J., & Álvarez, I. (2012). Physicochemical and functional properties of liquid whole egg treated by the application of Pulsed Electric Fields followed by heat in the presence of triethyl citrate. *Food research international*, 48(2), 484-490.
- Murphy, D. B. (2012). *Fundamentals of Light Microscopy and Electronic Imaging*. New York, NY: John Wiley & Sons.
- Murray, B. A., & FitzGerald, R. J. (2007). Angiotensin converting enzyme inhibitory peptides derived from food proteins: biochemistry, bioactivity and production. *Current pharmaceutical design*, 13(8), 773-791.
- Nakamura, S., & Kato, A. (2000). Multi- functional biopolymer prepared by covalent attachment of galactomannan to egg- white proteins through naturally occurring Maillard reaction. *Molecular Nutrition & Food Research*, 44(3), 201-206.
- Nakimbugwe, D., Masschalck, B., Anim, G., & Michiels, C. W. (2006). Inactivation of gram-negative bacteria in milk and banana juice by hen egg white and lambda lysozyme under high hydrostatic pressure. *International journal of food microbiology*, 112(1), 19-25.
- Nattress, F. M., Yost, C. K., & Baker, L. P. (2001). Evaluation of the ability of lysozyme and nisin to control meat spoilage bacteria. *International Journal of Food Microbiology*, 70(1), 111-119.
- Nattress, F. M., & Baker, L. P. (2003). Effects of treatment with lysozyme and nisin on the microflora and sensory properties of commercial pork. *International Journal of Food Microbiology*, 85(3), 259-267.
- Nayak, S. K., Arora, S., Sindhu, J. S., & Sangwan, R. B. (2006). Effect of chemical phosphorylation on solubility of buffalo milk proteins. *International dairy journal*, 16(3), 268-273.
- Neta, J. L. V., da Silva Lédo, A., Lima, A. A. B., Santana, J. C. C., Leite, N. S., Ruzene, D. S., ... & de Souza, R. R. (2012). Bromelain enzyme from pineapple: in vitro activity study under different micropropagation conditions. *Applied biochemistry and biotechnology*, 168(2), 234-246.
- Nilsang, S., Lertsiri, S., Supphantharika, M., & Assavanig, A. (2005). Optimization of enzymatic hydrolysis of fish soluble concentrate by commercial proteases. *Journal of food Engineering*, 70(4), 571-578.

- Nisbet, A. D., Saundry, R. H., Moir, A. J., Fothergill, L. A., and Fothergill, J. E. (1981). The complete amino-acid sequence of hen ovalbumin. *European Journal of Biochemistry*, 115, 335–345.
- Niu, F., Zhou, J., Niu, D., Wang, C., Liu, Y., Su, Y., & Yang, Y. (2015). Synergistic effects of ovalbumin/gum arabic complexes on the stability of emulsions exposed to environmental stress. *Food Hydrocolloids*, 47, 14-20.
- Niu, F., Niu, D., Zhang, H., Chang, C., Gu, L., Su, Y., & Yang, Y. (2016). Ovalbumin/gum arabic-stabilized emulsion: Rheology, emulsion characteristics, and Raman spectroscopic study. *Food Hydrocolloids*, 52, 607-614.
- Noh, D. O., & Suh, H. J. (2015). Preparation of egg white liquid hydrolysate (ELH) and its radical-scavenging activity. *Preventive nutrition and food science*, 20(3), 183.
- Norde, W. (2003). Colloids and interfaces in life sciences. New York: Marcel Dekker.
- Nys, Y., & Sauveur, B. (2004). The nutritional value of eggs. *INRA Prod. Anim*, 17(5), 385-393.
- Omana, D. A., Wang, J., & Wu, J. (2010). Ovomucin—a glycoprotein with promising potential. *Trends in food science & technology*, 21(9), 455-463.
- Osborn, H. T., & Akoh, C. C. (2004). Effect of emulsifier type, droplet size, and oil concentration on lipid oxidation in structured lipid-based oil-in-water emulsions. *Food Chemistry*, 84(3), 451-456.
- Ovissipour, M., Abedian, A., Motamedzadegan, A., Rasco, B., Safari, R., & Shahiri, H. (2009). The effect of enzymatic hydrolysis time and temperature on the properties of protein hydrolysates from Persian sturgeon (*Acipenser persicus*) viscera. *Food Chemistry*, 115(1), 238-242.
- Pacheco-Aguilar, R., Mazorra-Manzano, M. A., & Ramírez-Suárez, J. C. (2008). Functional properties of fish protein hydrolysates from Pacific whiting (*Merluccius productus*) muscle produced by a commercial protease. *Food Chemistry*, 109(4), 782-789.
- Padala, S. R., Williams, P. A., & Phillips, G. O. (2009). Adsorption of gum Arabic, egg white protein, and their mixtures at the oil– water interface in limonene oil-in-water emulsions. *Journal of agricultural and food chemistry*, 57(11), 4964-4973.
- Palazolo, G. G., Sobral, P. A., & Wagner, J. R. (2011). Freeze-thaw stability of oil-in-water emulsions prepared with native and thermally-denatured soybean isolates. *Food hydrocolloids*, 25(3), 398-409.
- Palumbo, M. S., Beers, S. M., Bhaduri, S., & Palumbo, S. A. (1996). Thermal resistance of *Listeria monocytogenes* and *Salmonella* spp. in liquid egg white. *Journal of Food Protection*, 59(11), 1182-1186.
- Paraskevopoulou, D., Boskou, D., & Paraskevopoulou, A. (2007). Oxidative stability of olive oil–lemon juice salad dressings stabilized with polysaccharides. *Food Chemistry*, 101(3), 1197-1204.
- Pavan, R., Jain, S., & Kumar, A. (2012). Properties and therapeutic application of bromelain: a review. *Biotechnology research international*, 2012.
- Pelegrine, D. H. G., & Gasparetto, C. A. (2005). Whey proteins solubility as function of temperature and pH. *LWT-Food Science and Technology*, 38(1), 77-80.
- Pernell, C. W., Foegeding, E. A., Luck, P. J., & Davis, J. P. (2002). Properties of whey and egg white protein foams. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 204(1), 9-21.

- Qian, C., & McClements, D. J. (2011). Formation of nanoemulsions stabilized by model food-grade emulsifiers using high-pressure homogenization: factors affecting particle size. *Food Hydrocolloids*, 25(5), 1000-1008.
- Qian, C., Decker, E. A., Xiao, H., & McClements, D. J. (2011). Comparison of biopolymer emulsifier performance in formation and stabilization of orange oil-in-water emulsions. *Journal of the American Oil Chemists' Society*, 88(1), 47-55.
- Qian, C., Decker, E. A., Xiao, H., & McClements, D. J. (2012). Physical and chemical stability of β -carotene-enriched nanoemulsions: Influence of pH, ionic strength, temperature, and emulsifier type. *Food Chemistry*, 132(3), 1221-1229.
- Raghavan, S., & Kristinsson, H. G. (2007). Conformational and rheological changes in catfish myosin as affected by different acids during acid-induced unfolding and refolding. *Journal of Agricultural and Food Chemistry*, 55(10), 4144-4153.
- Rahali, V., Chobert, J. M., Haertle, T., & Gueguen, J. (2000). Emulsification of chemical and enzymatic hydrolysates of β -lactoglobulin: characterization of the peptides adsorbed at the interface. *Molecular Nutrition & Food Research*, 44(2), 89-95.
- Raikos, V. (2010). Effect of heat treatment on milk protein functionality at emulsion interfaces. A review. *Food Hydrocolloids*, 24(4), 259-265.
- Raikos, V., Campbell, L., & Euston, S. R. (2007). Effects of sucrose and sodium chloride on foaming properties of egg white proteins. *Food Research International*, 40(3), 347-355.
- Rao, M. S., Chander, R., & Sharma, A. L. W. T. (2008). Synergistic effect of chitooligosaccharides and lysozyme for meat preservation. *LWT-Food Science and Technology*, 41(10), 1995-2001.
- Ray, M., & Rousseau, D. (2013). Stabilization of oil-in-water emulsions using mixtures of denatured soy whey proteins and soluble soybean polysaccharides. *Food research international*, 52(1), 298-307.
- Raybaudi-Massilia, R. M., Mosqueda-Melgar, J., Soliva-Fortuny, R., & Martín-Belloso, O. (2009). Control of pathogenic and spoilage microorganisms in fresh-cut fruits and fruit juices by traditional and alternative natural antimicrobials. *Comprehensive reviews in food science and food safety*, 8(3), 157-180.
- Robins, M. M., A. D. Watson, and P. J. Wilde. (2002). Emulsions-Creaming and rheology. *Current Opinion in Colloid & Interface Science*, 7 (5–6) 419–425.
- Roland, I., Piel, G., Delattre, L., & Evrard, B. (2003). Systematic characterization of oil-in-water emulsions for formulation design. *International journal of pharmaceutics*, 263(1), 85-94.
- Romero, A., Perez-Puyana, V., Marchal, P., Choplin, L., & Guerrero, A. (2017). Emulsification process controlled by a mixer type rheometer in O/W protein-based emulsions. *LWT - Food Science and Technology* 76, 26-32.
- Rousseau, D. (2002). Fat crystal behaviour in food emulsions. In A. G. Marangoni & S. S. Narine (Eds.). *Properties of lipids*. Available from http://www.foodnetbase.com/books/1526/DK1354_CH08.
- San Martín-González, M. F., Roach, A., & Harte, F. (2009). Rheological properties of corn oil emulsions stabilized by commercial micellar casein and high-pressure homogenization. *LWT-Food Science and Technology*, 42(1), 307-311.
- Sánchez, C. C., & Patino, J. M. R. (2005). Interfacial, foaming and emulsifying characteristics of sodium caseinate as influenced by protein concentration in solution. *Food Hydrocolloids*, 19(3), 407-416.

- Scherze, I., & Muschiolik, G. (2001). Effects of various whey protein hydrolysates on the emulsifying and surface properties of hydrolysed lecithin. *Colloids and Surfaces B: Biointerfaces*, 21(1), 107-117.
- Sebti, I., Chollet, E., Degraeve, P., Noel, C., & Peyrol, E. (2007). Water sensitivity, antimicrobial, and physicochemical analyses of edible films based on HPMC and/or chitosan. *Journal of agricultural and food chemistry*, 55(3), 693-699.
- Seekkuarachchi, I. N., Tanaka, K., & Kumazawa, H. (2006). Formation and characterization of submicrometer oil-in-water (O/W) emulsions, using high-energy emulsification. *Industrial & engineering chemistry research*, 45(1), 372-390.
- Silveti, T., Brasca, M., Lodi, R., Vanoni, L., Chiolerio, F., Groot, M. D., & Bravi, A. (2010). Effects of lysozyme on the microbiological stability and organoleptic properties of unpasteurized beer. *Journal of the Institute of Brewing*, 116(1), 33-40.
- Singh, A., & Dalgleish, D. (1998). The emulsifying properties of hydrolysates of whey proteins. *Journal of Dairy Science*, 81(4), 918-924.
- Singh, H., Ye, A., & Horne, D. (2009). Structuring food emulsions in the gastrointestinal tract to modify lipid digestion. *Progress in lipid research*, 48(2), 92-100.
- Singh, H. (2011). Aspects of milk-protein-stabilised emulsions. *Food Hydrocolloids*, 25(8), 1938-1944.
- Smith, D. M., & Culbertson, J. D. (2000). Proteins: functional properties. In G. L. Christen & J. S. Smith (Eds.), *Food chemistry: principles and applications* (pp. 131-138). West Sacramento: Science Technology Systems.
- Söderberg, J. (2013). Functional properties of legume proteins compared to egg proteins and their potential as egg replacers in vegan food. Retrieved from <https://stud.epsilon.slu.se>.
- Sourdet, S., Relkin, P., & César, B. (2003). Effects of milk protein type and pre-heating on physical stability of whipped and frozen emulsions. *Colloids and Surfaces B: Biointerfaces*, 31(1), 55-64.
- Sosa-Herrera, M. G., Berli, C. L. A., & Martinez-Padilla, L. P. (2008). Physicochemical and rheological properties of oil-in-water emulsions prepared with sodium caseinate/gellan gum mixtures. *Food Hydrocolloids*, 22(5), 934-942.
- Spellman, D., McEvoy, E., O'cuinn, G., & FitzGerald, R. J. (2003). Proteinase and exopeptidase hydrolysis of whey protein: Comparison of the TNBS, OPA and pH stat methods for quantification of degree of hydrolysis. *International Dairy Journal*, 13(6), 447-453.
- Srinivasan, M., Singh, H., & Munro, P. A. (1996). Sodium caseinate-stabilized emulsions: factors affecting coverage and composition of surface proteins. *Journal of Agricultural and Food Chemistry*, 44(12), 3807-3811.
- Srinivasan, M., Singh, H., & Munro, P. A. (1999). Adsorption behaviour of sodium and calcium caseinates in oil-in-water emulsions. *International Dairy Journal*, 9(3), 337-341.
- Srinivasan, M., Singh, H., & Munro, P. A. (2003). Influence of retorting (121 C for 15 min), before or after emulsification, on the properties of calcium caseinate oil-in-water emulsions. *Food chemistry*, 80(1), 61-69.
- Stadelman, W. J., & Cotterill, O. J. (Eds.), (1995). *Egg Science and Technology* (4th Ed.) New York: Food Products Press.

- Stein, P. E., Leslie, A. G. H., Finch, J. T. & Carrell, R. W. (1991). Crystal structure of uncleaved ovalbumin at 1.95Å resolution. *Journal of Molecular Biology*, 221, 941-959.
- Stevens, L. (1991). Egg white proteins. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*, 100(1), 1-9.
- Strixner, T., & Kulozik, U. (2011). Egg proteins. In *Handbook of food proteins* (pp. 150-209). Woodhead Publishing.
- Superti, F., Ammendolia, M. G., Berlutti, F., & Valenti, P. (2007). Ovotransferrin. In *Bioactive egg compounds* (pp. 43-50). Springer, Berlin, Heidelberg.
- Sun, C., & Gunasekaran, S. (2009). Effects of protein concentration and oil-phase volume fraction on the stability and rheology of menhaden oil-in-water emulsions stabilized by whey protein isolate with xanthan gum. *Food Hydrocolloids*, 23(1), 165-174.
- Tadros, T. F. (Ed.). (2013). *Emulsion formation and stability*. John Wiley & Sons.
- Talansier, E., Loisel, C., Dellavalle, D., Desrumaux, A., Lechevalier, V., & Legrand, J. (2009). Optimization of dry heat treatment of egg white in relation to foam and interfacial properties. *LWT-Food Science and Technology*, 42(2), 496-503.
- Tang, C. H., & Shen, L. (2013). Role of conformational flexibility in the emulsifying properties of bovine serum albumin. *Journal of agricultural and food chemistry*, 61(12), 3097-3110.
- Tavano, O. L. (2013). Protein hydrolysis using proteases: an important tool for food biotechnology. *Journal of Molecular Catalysis B: Enzymatic* 90 (2013); 1-11.
- Tcholakova, S., Denkov, N. D., Ivanov, I. B., & Campbell, B. (2006). Coalescence stability of emulsions containing globular milk proteins. *Advances in Colloid and Interface Science*, 123, 259-293.
- Tomczyńska-Mleko, M., Handa, A., Wesołowska-Trojanowska, M., Terpiłowski, K., Kwiatkowski, C., & Mleko, S. (2016). New controlled release material: aerated egg white gels induced by calcium ions. *European Food Research and Technology*, 242(8), 1235-1243.
- Thamnarathip, P., Jangchud, K., Jangchud, A., & Vardhanabhuti, B. (2016). Functional properties of protein hydrolysates from Riceberry rice bran. *International Journal of Food Science & Technology*, 51(5), 1110-1119.
- Thanasukarn, P., Pongsawatmanit, R., & McClements, D. J. (2004). Influence of emulsifier type on freeze-thaw stability of hydrogenated palm oil-in-water emulsions. *Food Hydrocolloids*, 18(6), 1033-1043.
- Tiwari, B. K., Valdramidis, V. P., O'Donnell, C. P., Muthukumarappan, K., Bourke, P., & Cullen, P. J. (2009). Application of natural antimicrobials for food preservation. *Journal of agricultural and food chemistry*, 57(14), 5987-6000.
- Turgeon, S. L., Gauthier, S. F., & Paquin, P. (1991). Interfacial and emulsifying properties of whey peptide fractions obtained with a two-step ultrafiltration process. *Journal of Agricultural and Food Chemistry*, 39(4), 673-676.
- Tsumura, K., Saito, T., Tsuge, K., Ashida, H., Kugimiya, W., & Inouye, K. (2005). Functional properties of soy protein hydrolysates obtained by selective proteolysis. *LWT-Food Science and Technology*, 38(3), 255-261.
- Van der Plancken, I., Van Loey, A., & Hendrickx, M. E. (2005). Combined effect of high pressure and temperature on selected properties of egg white proteins. *Innovative Food Science & Emerging Technologies*, 6(1), 11-20.

- Van der Plancken, I., Van Loey, A., & Hendrickx, M. E. (2006). Effect of heat-treatment on the physico-chemical properties of egg white proteins: A kinetic study. *Journal of Food Engineering*, 75(3), 316-326.
- Van der Plancken, I., Van Loey, A., & Hendrickx, M. (2007). Effect of moisture content during dry-heating on selected physicochemical and functional properties of dried egg white. *Journal of agricultural and food chemistry*, 55(1), 127-135.
- van der Ven, C., Gruppen, H., de Bont, D. B., & Voragen, A. G. (2001). Emulsion properties of casein and whey protein hydrolysates and the relation with other hydrolysate characteristics. *Journal of Agricultural and Food Chemistry*, 49(10), 5005-5012.
- Vanapalli, S. A., Palanuwech, J., & Coupland, J. N. (2002). Stability of emulsions to dispersed phase crystallization: effect of oil type, dispersed phase volume fraction, and cooling rate. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 204(1), 227-237.
- Vannini, L., Lanciotti, R., Baldi, D., & Guerzoni, M. E. (2004). Interactions between high pressure homogenization and antimicrobial activity of lysozyme and lactoperoxidase. *International Journal of Food Microbiology*, 94(2), 123-135.
- Visschers, R. W., & de Jongh, H. H. (2005). Disulphide bond formation in food protein aggregation and gelation. *Biotechnology advances*, 23(1), 75-80.
- Wall, J. S., & Huebner, F. R. (1981). Adhesion and cohesion.
- Walstra, P. (2003). *Physical chemistry of foods*. New York: Marcel Dekker.
- Walstra, P., & Van Vliet, T. (2008). Dispersed systems: basic considerations, In S. Damodaran, Parklin, K. L., & Fennema, O. R (ED.), *Fennema's Food Chemistry* (pp. 783-847). Boca Raton, FL: CRC Press.
- Watanabe, K., Shimoyamada, M., Onizuka, T., Akiyama, H., Niwa, M., Ido, T., & Tsuge, Y. (2004). Amino acid sequence of a-subunit in hen egg white ovomucin deduced from cloned cDNA. *DNA sequence*, 15(4), 251-261.
- Watkins, B. (1995). The nutritive value of the egg. In: Stadelman W, Cotterill O, (eds). *Egg Science and Technology*. New York: The Haworth Press Inc; p. 177- 94.
- Wang, J., Zhao, M., Yang, X., & Jiang, Y. (2006). Improvement on functional properties of wheat gluten by enzymatic hydrolysis and ultrafiltration. *Journal of Cereal Science*, 44(1), 93-100.
- Weijers, M., van de Velde, F., Stijnman, A., van de Pijpekamp, A., & Visschers, R. W. (2006). Structure and rheological properties of acid-induced egg white protein gels. *Food Hydrocolloids*, 20(2), 146-159.
- Wilde, P., Mackie, A., Husband, F., Gunning, P., & Morris, V. (2004). Proteins and emulsifiers at liquid interfaces. *Advances in Colloid and Interface Science*, 108, 63-71.
- Winuprasith, T., & Supphantharika, M. (2015). Properties and stability of oil-in-water emulsions stabilized by microfibrillated cellulose from mangosteen rind. *Food Hydrocolloids*, 43, 690-699.
- Wrolstad, R. E. & C. A. Culver (2008). *Colour Quality of Fresh and Processed Foods*. Washington, DC: American Chemical Society.
- Wu, J., & Acero-Lopez, A. (2012). Ovotransferrin: structure, bioactivities, and preparation. *Food Research International*, 46(2), 480-487.

- Wu, W. U., Hettiarachchy, N. S., & Qi, M. (1998). Hydrophobicity, solubility, and emulsifying properties of soy protein peptides prepared by papain modification and ultrafiltration. *Journal of the American Oil Chemists' Society*, 75(7), 845-850.
- Xiong, Z., Zhang, M., & Ma, M. (2016). Emulsifying properties of ovalbumin: Improvement and mechanism by phosphorylation in the presence of sodium tripolyphosphate. *Food Hydrocolloids*, 60, 29-37.
- Yao, X., Xu, Q., Tian, D., Wang, N., Fang, Y., Deng, Z., et al. (2013). Physical and chemical stability of gum arabic-stabilized conjugated linoleic acid oil-in-water emulsions. *Journal of agricultural and food chemistry*, 61(19), 4639-4645.
- Ye, A., Hemar, Y., & Singh, H. (2004). Enhancement of coalescence by xanthan addition to oil-in-water emulsions formed with extensively hydrolysed whey proteins. *Food Hydrocolloids*, 18(5), 737-746.
- Ye, A., & Singh, H. (2006). Heat stability of oil-in-water emulsions formed with intact or hydrolysed whey proteins: influence of polysaccharides. *Food Hydrocolloids*, 20(2), 269-276.
- Ye, A., Lo, J., & Singh, H. (2012). Formation of interfacial milk protein complexation to stabilize oil-in-water emulsions against calcium. *Journal of colloid and interface science*, 378(1), 184-190.
- Yin, L. J., Chu, B. S., Kobayashi, I. & Nakajima, M. (2009). Performance of selected emulsifiers and their combinations in the preparation of β -carotene nanodispersions. *Food Hydrocolloids*, 23(6), 1617-1622.
- Yin, C., Yang, L., Zhao, H., & Li, C. P. (2014). Improvement of antioxidant activity of egg white protein by phosphorylation and conjugation of epigallocatechin gallate. *Food research international*, 64, 855-863.
- Yuliana, M., Truong, C. T., Huynh, L. H., Ho, Q. P., & Ju, Y. H. (2014). Isolation and characterization of protein isolated from defatted cashew nut shell: Influence of pH and NaCl on solubility and functional properties. *LWT-Food Science and Technology*, 55(2), 621-626.
- Zhang, K., Li, Y., & Ren, Y. (2007). Research on the phosphorylation of soy protein isolate with sodium tripoly phosphate. *Journal of food engineering*, 79(4), 1233-1237.
- Zhang, T., Jiang, B., Mu, W., & Wang, Z. (2009). Emulsifying properties of chickpea protein isolates: Influence of pH and NaCl. *Food Hydrocolloids*, 23(1), 146-152.

Appendices

Appendix 1: Zeta potential for EWL solutions containing different protein concentrations (0.5-10% w/w) at different pH values using Zetasizer Nano ZS90

pH	Protein concentration (w/w %)									
	0.5	1	2	3	4	5	10			
2	33.4 ± 0.94	27.1 ± 3.24	30.9 ± 0.91	29.6 ± 1.52	30.7 ± 1.20	30.1 ± 0.46	30.6 ± 0.80			
3	31.0 ± 0.43	25.5 ± 0.6	27.1 ± 0.65	25.6 ± 1.28	21.3 ± 8.34	26.7 ± 1.01	28.9 ± 1.00			
4	22.9 ± 0.69	23.2 ± 0.87	21.1 ± 0.91	18.8 ± 2.89	18.7 ± 1.05	21.3 ± 0.31	23.6 ± 0.57			
5	2.34 ± 0.88	2.07 ± 0.71	-5.80 ± 2.33	-2.20 ± 0.87	-1.30 ± 0.35	1.90 ± 0.19	4.70 ± 0.52			
6	-5.32 ± 1.49	-8.18 ± 2.07	-10.3 ± 0.82	-16.7 ± 3.45	-13.4 ± 1.36	-10.7 ± 0.53	-18.2 ± 0.36			
7	-15.6 ± 2.65	-17.5 ± 0.93	-17.0 ± 0.74	-18.0 ± 0.48	-17.7 ± 0.42	-17.1 ± 0.55	-18.8 ± 0.10			
8	-17.5 ± 3.05	-20.2 ± 0.51	-18.6 ± 0.30	-19.0 ± 0.87	-21.1 ± 0.80	-20.7 ± 0.89	-20.4 ± 0.95			
(Original pH) 9.00	-20.4 ± 1.78	-24.0 ± 0.51	-21.1 ± 0.74	-19.4 ± 2.38	-24.1 ± 1.00	-24.2 ± 1.33	-25.6 ± 0.10			
10	-26.4 ± 1.31	-32.9 ± 0.77	-24.7 ± 0.91	-27.5 ± 0.96	-27.7 ± 1.06	-29.1 ± 0.21	-30.8 ± 0.75			
11	-33.2 ± 0.85	-26.9 ± 0.85	-30.0 ± 1.27	-31.4 ± 0.41	-33.2 ± 2.35	-31.1 ± 2.36	-34.8 ± 0.61			

Appendix 2: Turbidity for EWL solutions containing different protein concentrations (0.5-10% w/w) at different pH values using Zetasizer Nano ZS90

Protein concentration (%)							
pH	0.50%	1%	2%	3%	4%	5%	10%
3	0.179 ± 0.001 ^d	0.361 ± 0.004 ^d	0.692 ± 0.011 ^d	1.262 ± 0.001 ^d	1.582 ± 0.001 ^d	1.749 ± 0.002 ^d	2.243 ± 0.003 ^d
4	0.477 ± 0.004 ^b	1.120 ± 0.003 ^b	1.584 ± 0.011 ^b	1.723 ± 0.006 ^b	2.112 ± 0.005 ^b	2.251 ± 0.000 ^b	2.568 ± 0.004 ^b
5	0.867 ± 0.005 ^a	1.525 ± 0.001 ^a	1.745 ± 0.005 ^a	1.952 ± 0.005 ^a	2.234 ± 0.011 ^a	2.451 ± 0.000 ^a	2.589 ± 0.004 ^a
6	0.311 ± 0.004 ^c	0.810 ± 0.004 ^c	1.189 ± 0.010 ^c	1.665 ± 0.004 ^c	1.881 ± 0.006 ^c	2.112 ± 0.005 ^c	2.280 ± 0.006 ^c
7	0.150 ± 0.001 ^e	0.376 ± 0.008 ^e	0.500 ± 0.019 ^e	0.547 ± 0.005 ^e	0.628 ± 0.001 ^e	0.733 ± 0.002 ^e	0.990 ± 0.004 ^e
8	0.116 ± 0.004 ^f	0.272 ± 0.001 ^f	0.450 ± 0.003 ^f	0.531 ± 0.007 ^f	0.522 ± 0.008 ^f	0.536 ± 0.011 ^f	0.696 ± 0.007 ^f
9	0.071 ± 0.008 ^g	0.167 ± 0.008 ^g	0.188 ± 0.002 ^g	0.234 ± 0.002 ^g	0.251 ± 0.000 ^g	0.279 ± 0.001 ^g	0.332 ± 0.004 ^g
10	0.048 ± 0.001 ^h	0.083 ± 0.001 ^h	0.118 ± 0.002 ^h	0.155 ± 0.005 ^h	0.180 ± 0.001 ^h	0.199 ± 0.002 ^h	0.261 ± 0.000 ^h
p	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05

^{a-h} Means with different superscripts within a row are significantly different (p < 0.05).
Results are expressed as the means ± SD for n=6. Section 3.3.

Appendix 3: Time taken for EWL solution at 20°C to reach desired temperatures (57, 58, 59, 60 and 62°C).

Temperature	Time (minutes)
20	0
30	18
40	35
50	71
53	78
55	98
57	123
59	162
60	190

Appendix 4: Z-average and Zeta potential of O/W emulsions prepared with 5% (w/w) oil and various protein concentrations (0.1, 0.3, 0.5, 0.8, 1 and 2% w/w).

	Protein concentration (%)					
	0.1	0.3	0.5	0.8	1	2
Z-average (d.nm)	282.78 ± 4.54 ^c	242.08 ± 5.13 ^d	279.85 ± 7.59 ^c	319.48 ± 2.26 ^b	336.80 ± 20.54 ^b	703.68 ± 16.80 ^a
Zeta potential (mV)	-39.17 ± 2.81 ^a	-36.89 ± 1.20 ^{ab}	-35.33 ± 0.62 ^{ab}	-35.99 ± 0.66 ^{ab}	-37.37 ± 0.88 ^{ab}	-36.40 ± 0.34 ^{ab}

^{a-b} Means with different superscript within a row are significantly different (p < 0.05).
Results are presented as the means ± SD for n=3. Section 5.4.1

Appendix 5: Effects of heat treatment of temperature (60, 65, 70, 80 and 90°C) and holding time (0 and 30 minutes) on the mean particle size (Z-average (nm)) of emulsions prepared with different protein concentrations.

Protein concentration (%)		Time (minutes)	Temperature (°C)					p
		Control	60	65	70	80	90	
0.1	0	285.7 ± 5.15	274.1 ± 18.19	288.0 ± 5.01	277.7 ± 9.60	274.2 ± 16.40	264.6 ± 19.8	0.084
	30		285.7 ± 3.87	285.8 ± 3.00	288.2 ± 4.30	288.2 ± 3.50	273.9 ± 7.17	0.068
0.3	0	238.0 ± 12.70	242.7 ± 2.60	245.7 ± 5.90	243.9 ± 2.04	239.0 ± 2.04	245.5 ± 2.73	0.191
	30		245.4 ± 1.90	244.5 ± 1.67	245.4 ± 8.40	241.3 ± 3.90	247.3 ± 1.95	0.230
0.5	0	286.2 ± 4.90a	280.0 ± 5.86a	287.0 ± 5.90a	275.4 ± 3.31a	272.9 ± 3.32a	280.6 ± 2.93a	0.001
	30		280.2 ± 5.00a	275.6 ± 2.70a	280.7 ± 3.40a	276.2 ± 3.00a	279.2 ± 1.83a	0.01
0.8	0	324.8 ± 6.60a	337.7 ± 5.0a	282.0 ± 13.68	318.5 ± 12.65b	315.9 ± 3.22b	317.3 ± 6.48b	< 0.05
	30		248.2 ± 8.6d	272.4 ± 3.23c	270.4 ± 4.53c	315.0 ± 7.21b	324.6 ± 4.57a	< 0.05
1%	0	332.6 ± 7.20	341.6 ± 5.72ab	359.0 ± 2.32a	359.1 ± 7.10a	343.8 ± 1.20ab	337.7 ± 8.90ab	< 0.05
	30		308.8 ± 5.51c	358.1 ± 4.71a	342.7 ± 11.71ab	331.7 ± 8.62ab	319.8 ± 8.99b	< 0.05
2%	0	856.2 ± 20.0	752.3 ± 23.9c	746.0 ± 11.9c	751.4 ± 28.8c	834.2 ± 24.1b	869.7 ± 17.20ab	< 0.05
	30		738.0 ± 23.7cd	737.2 ± 24.1cd	789.2 ± 31.13b	876.9 ± 22.9ab	893.9 ± 12.83a	< 0.05

a-d Means the different superscript within a row are significantly different ($p < 0.05$). Results are presented as the means ± SD for n=6. Section 5.4.3.1

Appendix 6: Influence of CaCl₂ (0, 5, 10, 50 and 100 mM) on the mean particle size (D_{3,2}) of O/W emulsions prepared with 5% oil (w/w) and various EWP concentrations (0.1, 0.3, 0.5, 0.8, 1.0 and 2.0% w/w).

Protein concentration (%)	CaCl ₂ (mM)					
	0	5	10	50	100	P
0.1	0.31 ± 0.00d	3.12 ± 0.15c	3.27 ± 0.07c	4.62 ± 0.38b	7.52 ± 0.34a	<0.05
0.3	0.29 ± 0.03d	4.24 ± 0.11c	4.3 ± 0.02c	5.14 ± 0.08b	5.65 ± 0.28a	<0.05
0.5	0.32 ± 0.00c	5.70 ± 0.02b	5.55 ± 0.19b	5.92 ± 3.31b	7.52 ± 0.34a	<0.05
0.8	0.35 ± 0.03d	13.3 ± 0.21b	15.4 ± 0.21a	12.9 ± 0.14b	12.2 ± 0.23c	<0.05
1.0	0.36 ± 0.01e	10.21 ± 0.31b	12.76 ± 0.37a	5.46 ± 0.16c	2.53 ± 0.02d	<0.05
2.0	0.55 ± 0.08d	2.55 ± 0.06c	4.36 ± 0.32c	12.25 ± 0.26b	2.40 ± 0.06a	<0.05

a-e Means with different superscript within a row are significantly different (p < 0.05).
Results are presented as the means ± SD for n=6. Section 5.4.4.1

Appendix 7: Influence of NaCl (0, 50, 100, 200, 400 and 600 mM) on the mean particle size (D3,2) of O/W emulsions prepared with 5% oil (w/w) and various EWP concentrations (0.1, 0.3, 0.5, 0.8, 1.0 and 2.0% w/w).

Protein concentration (%)	NaCl (mM)					
	0	50	100	200	400	600
0.1	0.31 ± 0.00c	2.35 ± 0.03b	2.35 ± 0.03ab	4.46 ± 0.06ab	4.88 ± 0.35ab	7.12 ± 2.98a
0.3	0.29 ± 0.03d	3.44 ± 0.23c	4.08 ± 0.01bc	4.80 ± 0.17ab	5.57 ± 0.31a	5.65 ± 0.28a
0.5	0.32 ± 0.00d	3.48 ± 0.24c	4.09 ± 0.66bc	4.58 ± 0.32b	4.72 ± 0.26b	5.69 ± 0.02a
0.8	0.35 ± 0.03e	2.80 ± 0.20d	3.06 ± 0.03d	3.39 ± 0.14c	4.02 ± 0.07b	11.36 ± 0.13a
1%	0.36 ± 0.01c	0.51 ± 0.02c	1.01 ± 0.03b	1.08 ± 0.07b	1.14 ± 0.15ab	1.33 ± 0.10a
2%	0.55 ± 0.08c	0.63 ± 0.02b	0.68 ± 0.02ab	0.68 ± 0.03ab	0.71 ± 0.02a	2.40 ± 0.06a

^{a-d} Means with different superscript within a row are significantly different (p < 0.05).
Results are presented as the means ± SD for n=6. Section 5.4.4.1

Appendix 8: Colour specifications (L, a, b values) of emulsions prepared with original and hydrolysed egg white proteins.

Colour parameter	Type of emulsion	Protein concentration (%)			p
		1	5	10	
<i>L</i>	OEWP	91.17 ± 0.14 ^a	92.22 ± 0.27 ^a	92.12 ± 1.42 ^a	0.311
	EWPH	79.34 ± 0.08 ^a	70.11 ± 0.04 ^b	61.96 ± 0.05 ^c	< 0.05
<i>a</i>	OEWP	-0.04 ± 0.02 ^a	-1.11 ± 0.06 ^b	-1.98 ± 0.07 ^c	< 0.05
	EWPH	0.68 ± 0.01 ^c	1.43 ± 0.00 ^b	2.08 ± 0.04 ^a	< 0.05
<i>b</i>	OEWP	0.28 ± 0.05 ^c	6.28 ± 0.12 ^b	8.89 ± 0.02 ^a	< 0.05
	EWPH	5.32 ± 0.05 ^c	8.95 ± 0.01 ^b	9.57 ± 0.02 ^a	< 0.05

^{a-c} Means followed with different superscripts are significantly different (p < 0.05).

OEWP and EWPH represents original egg white protein and egg white protein hydrolysates respectively.

Results are expressed as mean ± standard deviation for n=6. Section 6.4.4.5