Research Article
Improving Emulsifying Properties of Egg White Protein by Partial Hydrolysis Combined with Heat Treatment

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Abstract: The present study investigated proteolysis combined with heat treatment to make hen Egg White (EW) an efficient emulsifier. EW was hydrolyzed by protease (Thermoase®) at various Enzyme Concentrations (EC) (w/w, 0.1%, 0.2%, 0.4%, 0.8%), followed by heating at 90°C for 8 min. Results showed that optimal emulsifying ability and stability, determined by measurement of emulsion turbidity, were obtained when EC was 0.4%, followed by heat treatment at 90°C. The hydrolysate thus prepared had higher emulsifying ability and stability than either native egg white (nEW) or small molecular weight EW peptides (Runpep®), close to the properties of Egg Yolk (EY) which was a reference as a food emulsifier. Surface hydrophobicity (H_0) was found to be linearly related to the emulsifying activity and stability of hydrolyzed egg white proteins.

Keywords: Egg white, emulsifying properties, heat treatment, hydrolysis, surface hydrophobicity

INTRODUCTION

Egg White (EW) is a significant protein source of dietary protein, accounting for about 58% of the entire mass of an egg, with a protein content of about 10% (Abeyrathne *et al*., 2013, Kovacs-Nolan *et al*., 2005). It is also known as a desirable ingredient for many foods such as bakery goods, meringues and meat products in which it is mainly used because of its excellent gelling and foaming properties (Alamprese *et al*., 2012). However, for some applications, it could be useful to improve and to diversify EW properties. In particular, increasing the emulsifying properties of EW could be an innovative way to obtain a pure protein emulsifier, which is a fat-free functional ingredient compatible with "light food" claims.

Enzymatic modifications are efficient for modifying protein functionality (Panyam and Kilara, 1996). Especially, proteolysis has been suggested as an efficient way to improve functional properties by Lqari *et al*. (2005). These authors showed that lupin protein and α-conglutin hydrolyzed using alkaline protease (alcalase) had better Emulsifying Activity (EA) than native lupin protein and α-conglutin, respectively. Although the Emulsifying Stability (ES) of hydrolysates of lupin protein and α-conglutin decreased relative to the native proteins, lupin protein hydrolysates were still thought to be potential to be used as ingredients in emulsion-based food formulations such as salad dressing and mayonnaise. Furthermore, thermal treatments that are usually used for inactivating the enzymes have also been shown to affect protein structure (Sanchez and Fremont, 2003), which should be related to protein functionality.

In the present study, measurements of EA and ES were performed for different hydrolyzed egg white (hEW), which were obtained by varying the EC and enzyme-inactivation temperature. Secondly, a relationship between H_0 versus EA and H_0 versus ES was attempted to be established.

MATERIALS AND METHODS

Preparation of egg white: Hen eggs were obtained from a local supermarket (Rennes, France) and were manually broken and separated into EY and EW. EW was mixed using a hand mixer (National MK-210, Japan) at a rotational speed of 540 rpm for 3s, then filtered by passing through a stainless mesh (sieve size 0.60 mm). Any foam was removed. Albumen pH was adjusted to 8.0 with 10% (w/v) citric acid solution before being used in the experiment.

Enzymatic hydrolysis of egg white: Thermoase PC10F (Amano Enzyme Inc., Japan) was added to EW at Enzyme Concentration (EC) of 0.1%, 0.2%, 0.4%

and 0.8% (w/w) after EW was warmed up to 55°C. Thermoase PC10F is a bacterial neutral metalloprotease extracted from *Geobacillus stearothermophilus*. It has an excellent thermal stability, with an optimum working temperature from 60 to 70°C. Enzymatic treatment was conducted as follows: at 55°C for 10 min, then heated up to 65°C and maintained at 65°C for 30 min before inactivation. Inactivation of the enzyme was achieved by holding the resulting hydrolysates at 90°C for 8 min, before homogenization with a mechanical homogenizer (IKA T18 basic, Germany) at Dial 5 (15,000 rpm) for 60 s. To ensure enzyme was completely inactivated, x-ray films (Fuji Film, Japan) were used. The surface of x-ray film is covered with thin gelatin film, which is hydrolyzed by the possible remained active enzyme, leading to the appearance of transparency of films.

Reference emulsifying peptide: Runpep® (Pharma Foods International Co. Ltd, Japan) is a mixture of EW peptides with molecular weight lower than 10 kDa (as reported in the product description), it was used as a reference for emulsifying properties. Runpep (80% proteins) was dissolved in distilled water at a concentration of 100 mg (protein)/mL as a reference sample, which was then stored at 4°C until use.

Determination of total protein content: Determination of total protein content in hEW, nEW, Runpep and EY was conducted by modified Lowry method (Lowry *et al*., 1951; Markwell *et al*., 1978)

Determination of hydrolysis degree: *h*EW obtained by using 0.1%, 0.2%, 0.4% and 0.8% EC without heating were stored at 4°C until the determination of the Degree of Hydrolysis (DH). Free amino groups were quantified using the o-phthalaldehyde (OPA) micro method described by Church *et al*. (1983), with modifications as described by Darrouzet-Nardi *et al*. (2013).

Surface hydrophobicity: Samples were diluted with phosphate buffer (0.01 M, pH 7.0) before centrifuging at 10,000 g for 10 min and supernatant of each sample was stored at 4°C for further analysis. Protein surface hydrophobicity (H_0) was measured using fluorescence probe ANS (1-anilinonaphthalene-8 sulfonic acid). ANS solution (45 μL, 8 mM) was added to 3 mL sample solution. ANS fluorescence intensity was measured at 470 nm with excitation at 390 nm. Excitation and emission slits were 2.5 nm. The slope of the plots of fluorescence intensity versus protein concentration (0, 0.05, 0.1, 0.15, 0.25 mg/mL) was calculated by linear regression and used as a measurement of H_0.

Determination of emulsifying properties: Emulsifying properties were measured according to the turbidimetric method developed by Pearce and Kinsella (1978) with slight modifications. Briefly, colza oil,

hEW (or EY, Runpep, nEW) and water were homogenized with a weight ratio of 3:2:1 by a mechanical dispenser (Polytron PT-MR2100, Switzerland) at 25,000 rpm for 1 min, then 200 μL of emulsion was pipetted from the bottom of the container immediately (T_0) and 2 h (T_{2h}) after homogenization. Each aliquot was diluted 1,000 times with SDS solution (0.1%, w/v). Absorbances of these diluted emulsions (A_0 and A_{2h}, respectively) were measured at 500 nm by a spectrophotometer (Unico S1205, USA). A_0 indicated emulsifying activity (EA). Emulsifying Stability (ES) was calculated as follows:

$$ES = A_0 / (A_0 - A_{2h})$$

Particle size measurement: hEW (or EY, Runpep, nEW) was diluted to the final protein concentration of 2% with deionized water. Then the protein dispersions were mixed with colza oil at volume ratios of 9:1, followed by pre-homogenizing for 2 min at 13,000 rpm using a dispenser (Polytron PT-MR2100, Switzerland) equipped with a 5 mm diameter head. The resulting emulsions were sealed and stored at 4°C until analysis. Droplet size distribution profiles of various freshly prepared emulsions were obtained with a laser diffraction particle size analyzer (Shimadzu, SALD-2200, Japan). Droplet size measurements are reported as the volume-average droplet size (Chang *et al*., 2016). All determinations were conducted on an individual sample in triplicates.

Statistics analysis: All experiments were carried out in triplicates. The data were subjected to multifactor Analysis of Variance (ANOVA), followed by the Least Significant Difference (LSD) test to determine the significant difference between samples at $p < 0.05$ using the software SPSS V.16.

RESULTS AND DISCUSSION

Degree of hydrolysis: To study the effect of DH on the emulsifying properties of hEW, hydrolysis using various EC was carried out. As shown in Table 1, DH increased significantly when EC increased from 0.1% (DH of 6.5%) to 0.4% (DH of 11.6%). After an additional increase up to 0.8% EC, more extensive degradation occurred. Moreover, the commercial product Runpep® was two times more hydrolyzed (DH of 26.0%) than the most hydrolyzed samples prepared in the present study (DH of 12.7%).

Emulsifying properties: The ability of a protein to aid the formation of an emulsion is related to its ability to attach to and stabilize the oil-water interface, the more the interfacial area that can be coated by the available protein, EA should be higher (Day *et al*., 2009). Due to the formation of smaller droplets during emulsification, more light scattering resulted in higher turbidity and the

Table 1: The degree of hydrolysis (DH) and surface hydrophobicity (H$_0$) of egg white hydrolysates obtained by using various enzyme concentration (0.1%, 0.2%, 0.4%, 0.8%), compared to Runpep: highly hydrolyzed commercial egg white peptides

		hEW				nEW	Runpep
		0.1% EC	0.2% EC	0.4% EC	0.8% EC		
DH (%)		6.52±0.20[e]	7.78±0.39[d]	11.11±1.60[c]	12.74±0.59[b]	N/A	26.76±0.26[a]
H$_0$	Without heating	167.96±3.07[ab]	172.60±9.34[ab]	164.80±1.29[b]	129.56±9.16[c]	107.61±2.93[d]	76.34±3.40[e]
	Heated at 90°C	168.40±9.42[ab]	177.45±6.09[a]	180.86±2.44[a]	180.35±7.99[a]	N/A	N/A

N/A means data was not analyzed. Means with different letters are significantly different (p<0.05)
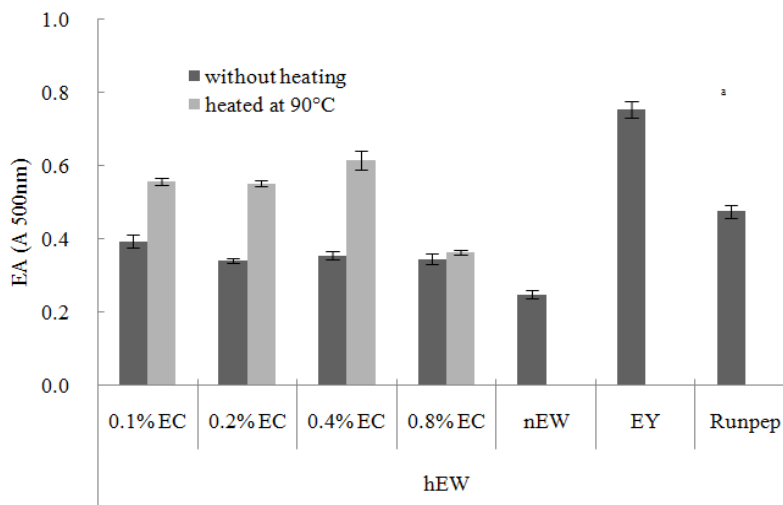

Fig. 1: Comparison of Emulsifying Activity (EA) of different samples. (90°C indicates enzyme inactivation temperature for hydrolysate, without heating represents hydrolysate without enzyme inactivation. nEW: Native Egg White; hEW: Hydrolyzed Egg White; EC: Enzyme Concentration; EY: Egg Yolk; Runpep: highly hydrolyzed commercial egg white peptides. Means with different letters are significantly different (p<0.05)

turbidity increase indicates an increase in EA (Van Vliet *et al*., 2002). Similarly, the maintenance of a high turbidity value during the storage of an emulsion indicates high ES, while a turbidity decrease indicates instability of the emulsion.

Turbidity measurements of emulsions stabilized by different hydrolysates were performed immediately after emulsification (T$_0$) and after 2 h of storage (T$_{2h}$). Absorbance (500 nm) observed at T$_0$ was used as an index of EA, ES was calculated by using the equation in the method. Results of EA and ES were shown in Fig. 1 and 2, respectively. Results showed that among all the hydrolysates, hEW obtained using an EC at 0.4% combined with a heat treatment at 90°C resulted in the best EA and ES, which was comparable to that of EY and much higher than that of nEW. Furthermore, regarding EA, almost all the hEW samples were better than nEW. ES of all the EW samples without heating was such a small value (close to 1), that meansturbidity of emulsions after 2 h became almost 0, emulsions separated completely. However, after heating, ES increased, especially for hEW obtained with an EC at 0.4% when heated at 90°C. It is noticeable that for hEW obtained with an EC at 0.1%, 0.2% and 0.4%, heating at 90°C contributed to the improvement of EA and ES, while for hEW obtained with an EC at 0.8%, heating at 90°C only contributed to the increase of ES, without

any effect on EA. In fact, beyond ECat 0.4% when heated at 90°C, EA and ES decreased when EC increased. This could suggest that the higher emulsifying properties are obtained for moderate proteolysis. And the highly hydrolyzed product Runpep (DH of 26%) offered an excellent EA but low ES. It can thus be concluded that limited proteolysis (DH<12.7%), as well as heat treatment after proteolysis, can result in the improvement of EA and ES.

It is well known that protein hydrolysates can be attached to the oil-water interface more efficiently compared to proteins, because of molecular size. However, protein hydrolysates are more difficult to form a network structure due to fewer hydrophobic binding sites (Pokora *et al*., 2013), resulting in a relative worse ES of protein hydrolysates. Because the complex, folded and coiled protein molecules were cut down into separate units by the previous hydrolysis treatment, the hydrolysate after heating at 90°C was unable to form a well ordered tertiary network or matrix, resulting in a creamy texture, without causing any gelation or coagulation even heated at 90°C.

The average droplet size, the difference between the maximum and minimum diameter of droplets of the dispersed phase and the degree of their dispersion are considered as the significant parameters characterizing a given emulsion (Dajnowiec *et al*., 2016). The droplet size distribution influences the properties of the

emulsion in aspects such as degradation rates, long-term stability, texture and visual appearance (Jurado *et al.*, 2007; Fernandez *et al.*, 2004). The particle size was proved to be an indication of the emulsifying capacity of the hydrolysates and provides information about the tendency of the emulsions to coagulate or coalesce. And droplets dispersed (oil) should be small enough to remain in suspension and should be evenly distributed throughout the matrix (Rahmati *et al.*, 2014).
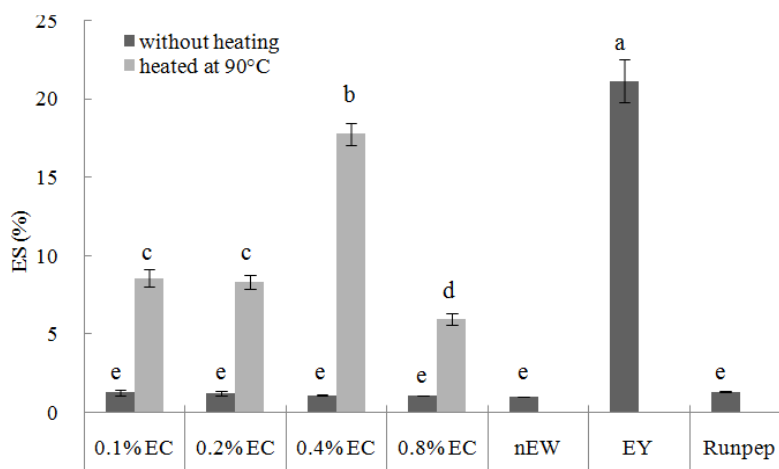

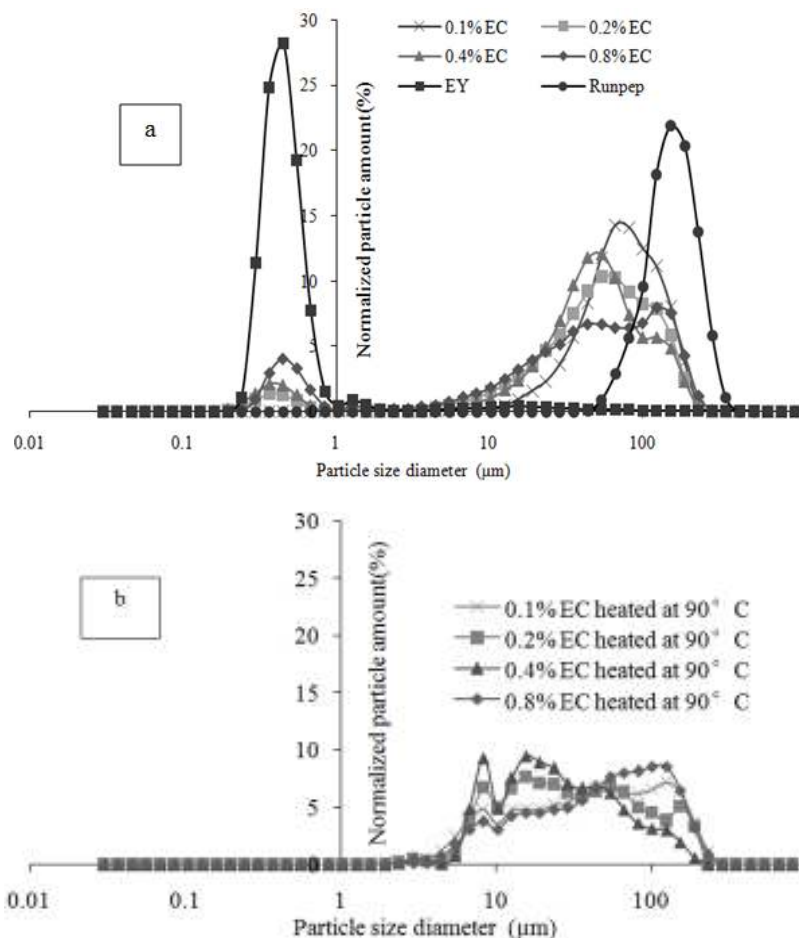Fig. 2: Comparison of Emulsifying Stability (ES) of different samples (Fig. 1)

Fig. 3: Particle size distribution of emulsion containing 10% oil and different emulsifiers (Fig. 1)

In the current study, particle size was also used to evaluate the emulsifying properties. Droplet size distribution curves of hEW samples without heating were shown in Fig. 3a. According to the shape of curves (Fig. 3a), EY and Runpepexhibited single peaked droplet size distribution, the amount of small droplet size (between 0.10 and 1 μm) was found to be the most in EY. The smallest and the largest particle size were found in EY and Runpep respectively, referring to the low emulsifying property of Runpep compared to EY. This result is in accordance with that obtained by the former turbidimetric method. Regarding hEW, all the hEW samples exhibited double peaked droplet size distribution, the peak of the curve for hEW obtained at EC of 0.1% was found to be closer to the right side of the x-axis, which revealed a higher average droplet size than the other hEW samples. The average particle sizes of each hEW samples were observed between the size of EY and Runpep. Regarding hEW heated at 90°C, shape of droplet size distribution curves (Fig. 3b) became complicated than hEW without heating, all the heated hEW samples showed a same tendency and situated more on the left side of the x-axis, which suggested that heat at 90°C contributed to providing smaller droplet size of emulsions. A peak at the point of size less than 10 μm was found for all the hEW samples. Double peaks were observed for hEW obtained at EC of 0.4% heated at 90°C and both of these two peaks were found to situate at a size around 10 μm, showing a relative smaller mean size than the other samples. EW obtained at an EC of 0.4% heated at 90°C was also observed to possess a good emulsifying property by the turbidimetric method as shown before.

Surface hydrophobicity measurement: Measurement of surface hydrophobicity (H_0) of hEW is shown in Table 1. H_0 was reported to have a great significance in elucidating the protein function (Kato and Nakai, 1980). It is revealed that, compared with nEW, H_0 became much higher after hydrolysis when EC was 0.2%. However, as shown by Runpep and hEW obtained at EC of 0.8%, highly hydrolyzed EW had fewer hydrophobic binding sites than large peptides.

Heat treatment was performed to inactivate the enzyme. As suggested by Kato *et al*. (1981), H_0 of ovalbumin and lysozyme significantly increases with progressive heating. In this study, heated hEW samples showed a higher H_0 than that of hEW without heat, probably due to the exposure of some hydrophobic groups after heating. In an aggregating-limited temperature (less than 90°C), higher temperature contributes to the exposition of hydrophobic groups initially buried in the core of proteins. But physicochemical changes caused by heat treatment (insolubility, 2D-and 3D-structure modifications, etc) also play an essential role in the determination of H_0. Thus, it is complicated to evaluate the individual effect
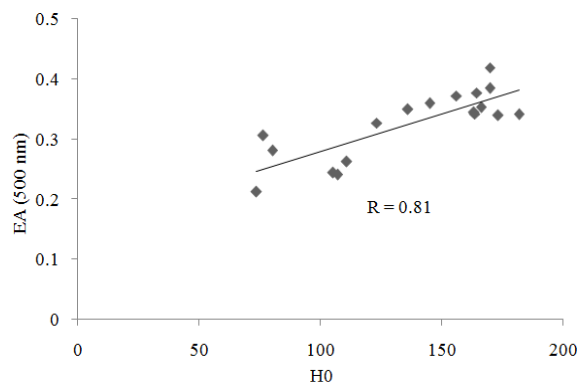

Fig. 4: Relationship between surface hydrophobicity and emulsifying activity
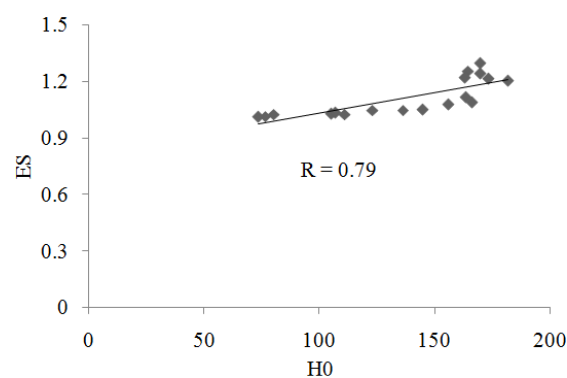

Fig. 5: Relationship between surface hydrophobicity and emulsifying stability

of hydrolysis and heat treatments on H_0 of hEW. In the present study, we attempted to establish a relationship between H_0 versus EA as well as H_0 versus ES, as shown in Fig. 4 and 5, respectively. It is noticeable that a good correlation could be determined for H_0 versus EA (R = 0.81) and H_0 versus ES (R = 0.79), i.e., the higher the surface hydrophobicity, EA and ES should be higher.

Thus, it can be concluded that H_0 plays a significant role in EA and ES. H_0can be changed by DH, DH would be a relevant complementary parameter; Furthermore, viscosity, as well as solubility may also be necessary factors affecting the emulsifying properties of EW proteins.

CONCLUSION

The effects of enzyme concentration and heat treatment on the degree of hydrolysis, surface hydrophobicity and emulsifying properties of egg white proteins were evaluated. By using various enzyme concentrations and different heat treatment temperature, we could prepare egg white hydrolysates that were all more efficient than native egg white considering emulsifying activity and stability. The optimal results were obtained when enzyme concentration was 0.4% (DH of 11.6%), combined with heat treatment at 90°C.

Higher hydrolysis (DH of 26.0%) resulted in peptides which are with an excellent emulsifying property but low emulsifying stability. Heating at 90°C after proteolysis contributed to the improvement of the emulsifying properties of egg white hydrolysates. Surface hydrophobicity was found to be linearly related to the emulsifying activity and stability of hydrolyzed egg white proteins.

REFERENCES

Abeyrathne, E.D., H.Y. Lee and D.U. Ahn, 2013. Egg white proteins and their potential use in food processing or as nutraceutical and pharmaceutical agents--a review. Poult. Sci., 92(12): 3292-3299.

Alamprese, C., E. Casiraghi and M. Rossi, 2012. Foaming, gelling and rheological properties of egg albumen as affected by the housing system and the age of laying hens. Int. J. Food Sci. Tech., 47(7): 1411-1420.

Chang, C., F. Niu, L. Gu, X. Li, H. Yang, B. Zhou *et al.*, 2016. Formation of fibrous or granular egg white protein microparticles and properties of the integrated emulsions. Food Hydrocolloid., 61: 477-486.

Church, F.C., H.E. Swaisgood, D.H. Porter and G.L. Catignani, 1983. Spectrophotometric assay using o-phthaldialdehyde for determination of proteolysis in milk and isolated milk proteins [1]. J. Dairy Sci., 66(4): 1219-1227.

Dajnowiec, F., P. Banaszczyk, A. Kubiak, M. Biegaj and L. Zander, 2016. The study on oil droplet size distribution in O/W emulsions prepared by the use of the asymmetric membrane. Pol. J. Natur. Sc., 31(4): 665-680.

Darrouzet-Nardi, A., M.P. Ladd and M.N. Weintraub, 2013. Fluorescent microplate analysis of amino acids and other primary amines in soils. Soil Biol. Biochem., 57: 78-82.

Day, L., M. Xu, L. Lundin and T.J. Wooster, 2009. Interfacial properties of deamidated wheat protein in relation to its ability to stabilise oil-in-water emulsions. Food Hydrocolloid., 23(8): 2158-2167.

Fernandez, P., V. André, J. Rieger and A. Kühnle, 2004. Nano-emulsion formation by emulsion phase inversion. Colloid Surface A, 251(1-3): 53-58.

Jurado, E., V. Bravo, F. Camacho, J.M. Vicaria and A. Fernández-Arteaga, 2007. Estimation of the distribution of droplet size, interfacial area and volume in emulsions. Colloid Surface A, 295(1-3): 91-98.

Kato, A. and S. Nakai, 1980. Hydrophobicity determined by a fluorescence probe method and its correlation with surface properties of proteins. BBA-Protein Struct., 624: 13-20.

Kato, A., N. Tsutsui, N. Matsudomi, K. Kobayashi and S. Nakai, 1981. Effects of partial denaturation on surface properties of ovalbumin and lysozyme. Agric. Biol. Chem., 45: 2755-2760.

Kovacs-Nolan, J., M. Phillips and Y. Mine, 2005. Advances in the value of eggs and egg components for human health. J. Agr. Food Chem., 53: 8421-8431.

Lqari, H., J. Pedroche, J. Girón-Calle, J. Vioque and F. Millán, 2005. Production of lupinus angustifolius protein hydrolysates with improved functional properties. Grasas Aceites, 56: 135-140.

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J., Randall, 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem., 193(1): 265-275.

Markwell, M.A.K., S.M. Haas, L.L. Bieber and N.E. Tolbert, 1978. A modification of the lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal. Biochem., 87(1): 206-210.

Panyam, D. and A. Kilara, 1996. Enhancing the functionality of food proteins by enzymatic modification. Trends Food Sci. Tech., 7(4): 120-125.

Pearce, K.N. and J.E. Kinsella, 1978. Emulsifying properties of proteins: Evaluation of a turbidimetric technique. J. Agr. Food Chem., 26(03): 716-723.

Pokora, M., E. Eckert, A. Zambrowicz, Ł. Bobak, M. Szołtysik *et al.*, 2013. Biological and functional properties of proteolytic enzyme-modified egg protein by-products. Food Sci. Nutr., 1(2): 184-195.

Rahmati, K., T.M. Mazaheri and K. Daneshvar, 2014. Soy milk as an emulsifier in mayonnaise: Physico-chemical, stability and sensory evaluation. J. Food Sci. Technol., 51: 3341-3347.

Sanchez, C. and S. Fremont, 2003. Consequences of heat treatment and processing of food on the structure and allergenicity of component proteins. Rev. Fr. Allergol., 43: 13-20.

Van Vliet, T., A.H. Martin and M.A. Bos, 2002. Gelation and interfacial behaviour of vegetable proteins. Curr. Opin. Colloid In., 7(5-6): 462-468.